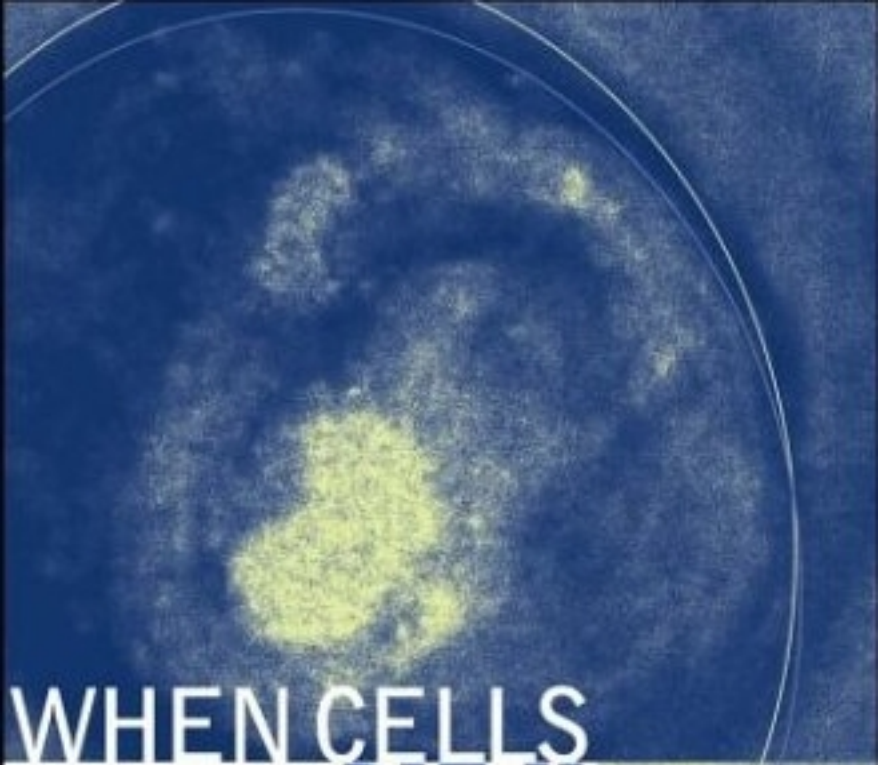

# WHEN CELLS DIE II

## A COMPREHENSIVE EVALUATION of APOPTOSIS and PROGRAMMED CELL DEATH

Edited by
Richard A. Lockshin
Zahra Zakeri

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## A Comprehensive Evaluation of Apoptosis and Programmed Cell Death


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# Preface

In this new edition of *When Cells Die*, we have tried to provide both a background to the subject of programmed cell death/apoptosis and an introduction to the most interesting new areas in the field. We hope our readers will find that we have provided a useful and coordinated work, and that it helps to introduce a new generation to this exciting field. We acknowledge the wonderful efforts of many earlier and current researchers, and we also wish to make the following remarks:


- This book is dedicated to the memory of Samuel and Florence Lockshin, who would have taken great pleasure in seeing how far this topic has grown.
- This volume is further dedicated to all the children who grew up with the nickname "questions"—may they enjoy science as much as we have—and to the parents who encouraged their questions.
- RAL also acknowledges, with affection, all his relatives who asked, "You're *still* working on the same question? Haven't you answered it yet?"
- ZZ thanks her parents Pari and Sirous Nabavi for fostering her enthusiasm to look for answers.
- ZZ acknowledges her teachers and students who have taught her to love exploration.
- We both thank our friends and family who tolerated our missing numerous occasions.
- We thank the numerous students who critiqued, commented on, and chased down references for this book.
- Both RAL and ZZ enjoyed support from the National Institutes of Health during the preparation of this work, and some of the results reported in the chapters here derive from that support.
- We especially dedicate this book to all the developmental and cell biologists who for so long quietly worked on the topic, sometimes against all popular odds.

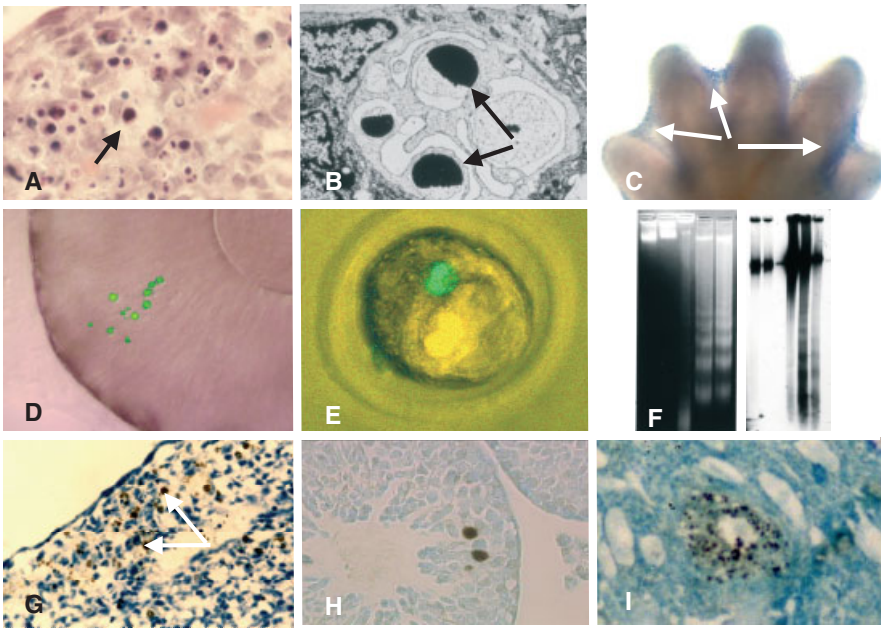


PLATE 2.1 Examples of several techniques to illustrate cell death or apoptosis. (A) Light microscopy image of haematoxylin and eosin staining of interdigital region of a day 13.5 embryonic mouse hand palette. Darkly stained cells (arrow) are easily distinguished but must be examined at high magnification to confirm that they are not metaphase cells. (B) Apoptotic nuclei (arrows) are readily recognized in the vacuoles of phagocytes (M) in this electron micrograph. (C) Nile blue sulfate is readily taken up into dead cells (actually vacuoles of phagocytes) in the interdigital regions of a day 12.5 embryonic mouse hand palette. Some of the most prominent regions are indicated by arrows. (D) Acridine orange penetrates the stage 17 zebrafish eye. Dead cells in the lens are easily identified by green fluorescence. The phase and fluorescence images were overlaid. (E) Annexin V coupled to a fluor marks the exposed phosphatidylserine on one cell of a mouse blastocyst, as is readily seen by fluorescence using confocal microscopy. The bright green cell is annexin-positive. (F) DNA fragmentation detected by gel electrophoresis using conventional gel (left) and end labeling of the fragmented DNA (right). In both figures, there are two control lanes to the left, and the rightmost lanes are DNA from cells undergoing apoptosis. The ladder seen at lower molecular weights represents fragments of DNA differing in size by 180 bp, indicating that the DNA was cut between nucleosomes. (G) The TUNEL technique, here using the brown DAB-peroxidase reaction revealing dead cells, marked by arrows, in the interdigital regions of a day 13.5 mouse hand palette. (H) DNA fragmentation using TUNEL showing cell death (dark brown cells, TUNEL positive) in the seminiferous tubules of the adult testis. (I) DNA fragmentation using TUNEL showing cell death (dark brown cells) in granulosa cells of atretic follicles of an adult mouse ovary.

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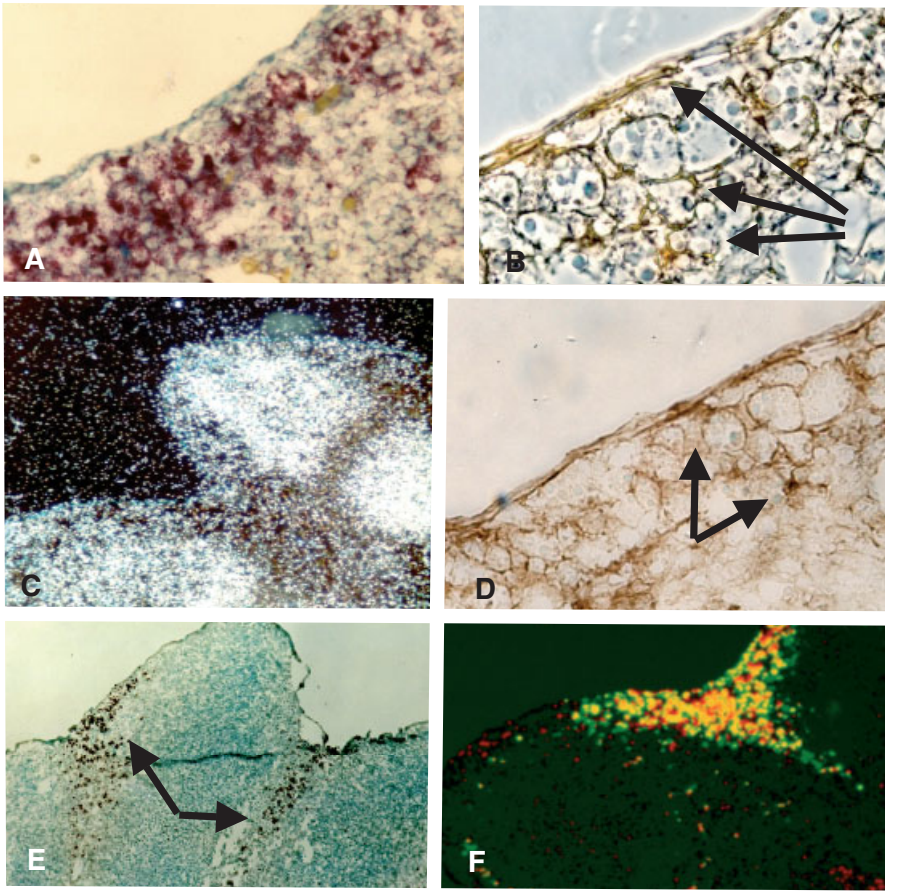


PLATE 2.2 Further examples of techniques to illustrate cell death or apoptosis, continued. (A) Interdigital region of the mouse hand palette has been incubated in the presence of a naphthol AS-BT phosphate substrate. The magenta-to-purple color marks high acid phosphatase activity. (B) F4/80 antibody identifies the cell membrane of a macrophage, as indicated by the arrows, in a mouse embryo hand palette. (C) In situ hybridization for bcl-2 message in a developing mouse digit reveals substantial labeling over the cartilaginous areas, but there is no difference in the level of labeling in the interdigital region in which cell death is present compared to the adjacent regions, suggesting that there is no change in the level of Bcl-2 in this region. (D) Immunohistochemistry reveals upregulation of transglutaminase protein (arrows) in dying cells in a mouse hand palette. Transglutaminase is often a good marker for cell death (Piacentini et al., 1991). (E) Although most proteins do not change much in amount, immunohistochemistry can reveal activation of enzymes such as Cdk5, illustrated here (arrows), from a day 13.5 mouse embryo hand palette. (F) Double staining of DNA fragmentation (TUNEL, green) and Cdk5 protein (red) merged (yellow) in a confocal image of a section of the interdigital region of a day 13.5 mouse hand palette.

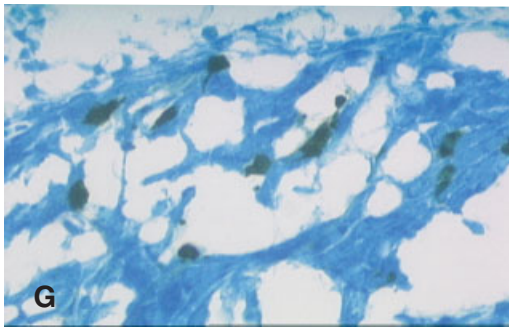


PLATE 2.2 (continued) (G) Using immunohistochemistry to show the activation of caspase 3 using an antibody specific to the active form. The dark staining reveals the activation of caspase 3 in the gestation day 13.5 mouse liver.

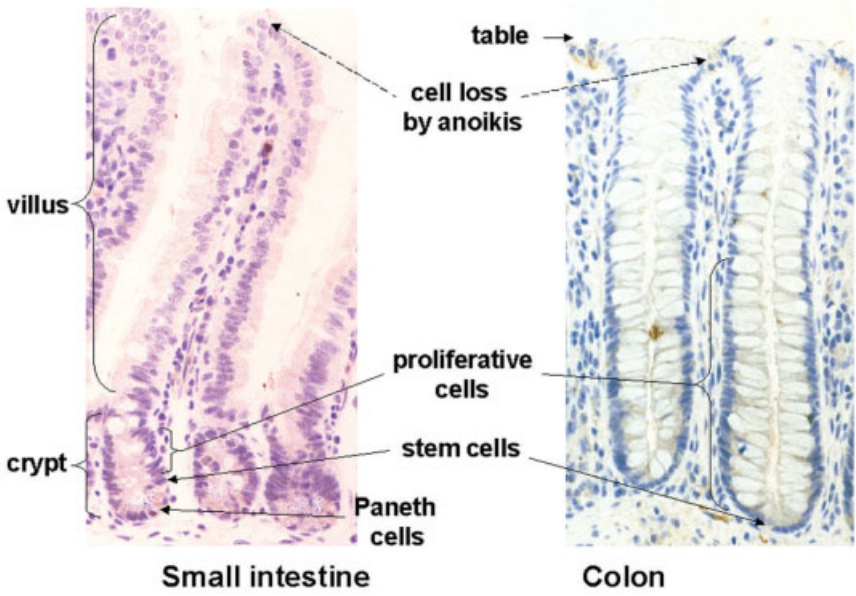


PLATE 9.1 The intestinal epithelium.

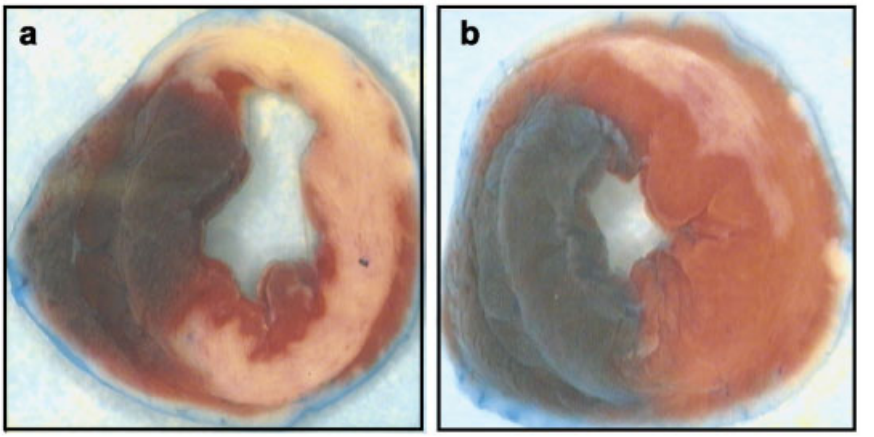


PLATE 19.3A Example of reduction of myocardial infarct size during ischemia-reperfusion in Fas-deficient *lpr* mice. *Lpr* and wild-type mice were subjected to 30 minutes of left anterior descending coronary artery ligation followed by 24 hours of reperfusion. Following sacrifice, the region at risk (or ischemic zone) is identified by religating the coronary artery and infusing Evans blue dye. The absence of blue demarcates the region at risk. The heart is also incubated with 2,3,5-triphenyltetrazolium chloride (TTC), a measure of mitochondrial reductases, to assess viable tissue (red). Within the nonblue zone, the infarct is demarcated by the absence of red, which appears white. Panel A shows typical Evans blue/TTC staining of a wild type (a) and an *lpr* (b) heart. Note that, despite similar regions at risk (nonblue), the wild-type heart exhibits a much larger infarct (white) than the *lpr* heart. See color insert. Panel B shows the quantitative analysis for nine wild-type and eight *lpr* mice.

SECTION

I

# CELL DEATH ORIGIN AND PROGRESSION

CHAPTER

1

# INTRODUCTION

RICHARD A. LOCKSHIN AND ZAHRA ZAKERI

We are all transients in the enterprise of discovery. The 2002 Nobel Prize in medicine and physiology, for the development of studies of *Caenorhabditis*, included a substantial salute to the elucidation of the genetics of cell death—a brilliant series of experiments and interpretations, but one that had many precedents and will have many descendants.

The tale of cell death can be described as several stages. The first was the discovery of cell death and its recognition. The second was the surveying and categorizing of types and distribution of cell death. The third began the analysis of the physiological controls and mechanisms. We are now in the fourth phase, in which analysis of the genetics of cell death mechanisms is teaching us about mechanisms, means, purposes, and controls. Many mechanisms have been established, but the normal physiological controls (as opposed to responses of cells exposed to toxins or cells that have been altered by knockout or upregulation of individual genes) are less well understood. For instance, many cancers have as a major component the failure of the cancerous cells to undergo apoptosis on schedule. However, in most of these cells, the machinery of apoptosis remains intact, but the threshold at which it is activated increases. This threshold depends on many parameters deriving from the prior history of the cell, its metabolism, its stage of cell cycle, and growth factors (**Sang and Giordano***), as well as the matrix in

*Authors of chapters in this book have been boldfaced.

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which cells are embedded (**Ishizuya-Oka et al.**). Thus the fifth phase, now beginning, will address the application of knowledge of cell death to the manipulation of cell death in clinical situations. Beyond that phase, scientific predictions are too unreliable to venture.

To establish common ground, it is most useful to define our terms. The first section, therefore, consists of a brief outline of apoptotic and other cell deaths. This understanding allows us to address the history of the field and, finally, the theoretical but very important question of the evolution of cell death mechanisms.

## Types of Cell Death

There are many types of cell death (Fig. 1) defined by morphological or biochemical behavior of the cell. Severely injured cells may undergo necrosis. Physiological deaths include the best known form, apoptosis. There are two variants of apoptosis, described below, but physiologically cells also die by autophagic mechanisms, as **Bursch et al.** describe. Plant cells, as **Mittler and Cheung** discuss, osteoblasts, intestinal enterocytes (**Wilson and Potten**), and other cells constrained by their environment may undergo

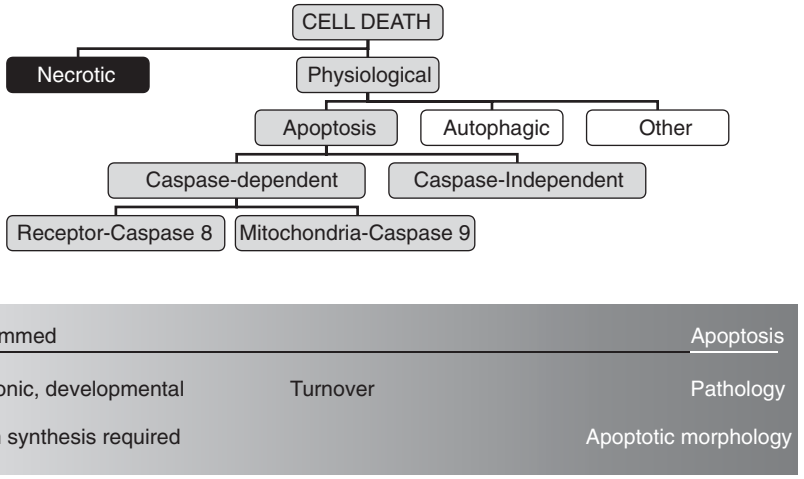


FIGURE 1. Classifications of cell death. Most cell deaths in the body are under physiological control. Necrotic deaths are presumed not to be controlled. The paradigm, and best understood version, for physiological cell death is caspase-dependent apoptosis, but as described in the text, there are several other forms. Programming is most apparent in developmental situations, and classical apoptosis is most clearly seen in experimental and pathological situations. Turnover is presumed to be apoptotic but is not well documented.

various forms of degeneration resulting from the failure of the dying cells to be consumed by phagocytes. It is also possible for cells to manifest various intermediate forms. Each of these general types is described below.

## Apoptosis

Apoptosis was first defined by its morphological criteria (Fig. 2) and then by the particular, limited form of degradation of DNA. Most of these characteristics are controlled by the activation of restricted-target proteases called caspases, but there are forms of apoptosis that are considered to be independent of caspase activation.

## Caspase-dependent Cell Death (initiated by an extracellular signal)

Paradigmatic apoptosis is substantially explained by the activation of a cascade of site-specific proteases, the caspases (cysteinyl proteases cleaving C-terminal to an aspartic acid) and the activation of proteases and nucleases in the nucleus to destroy chromatin and, finally, DNA. Caspases are further characterized by their active site, QACXG. These changes lead to the hallmarks of apoptosis, namely: condensation and blebbing of the cytoplasm; condensation and margination of chromatin against the nuclear membrane; internucleosomal degradation of DNA as determined by electrophoresis (DNA ladder), FACS analysis (subdiploid DNA/cell), or TUNEL labeling; and exteriorization of phosphatidylserine. The caspase family was discovered relatively recently (Yuan et al., 1993) but proves to be highly conserved from *Caenorhabditis* and *Drosophila* to mammals (**Dorstyn and Kumar**). The sequences of apoptosis will be illustrated in a bit, following a brief discussion of the two major modes of activation of caspases.

Caspases are subdivided into two general categories: initiator caspases (characterized by long prodomains) and effector caspases. The effector caspases, typified by caspase 3 and caspase 7, are those that attack critical cytoplasmic proteins, such as cytoskeletal proteins, polyadenosylribose polymerase, and other strategic enzymes or structural proteins. The effector caspases typically exist in proenzyme form in the cytoplasm and are proteolytically activated by the initiator caspases, typified by caspases 8 and 9. These two caspases are activated in different manners. Caspase 8 is activated at the cell membrane. When a member of the TNF-α family including TNF-α and Fas-ligand binds to its appropriate receptor, through a specific intracellular sequence on the receptor molecule (the death receptor, DD) and, in conjunction with several other molecules (the death-inducing signaling complex, DISC), it recruits and activates caspase 8 by hydrolyzing the inactivating peptide from the proenzyme (Fig. 3).

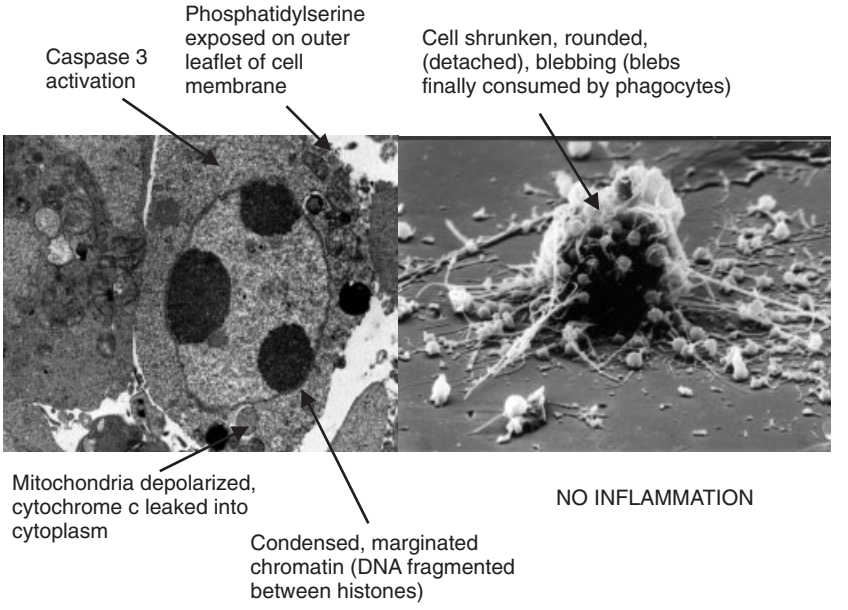


FIGURE 2. Characteristics of apoptosis. A. Left: transmission electron micrograph of staurosporine-induced apoptosis in a lymphoid cell. Right: scanning electron micrograph of a human keratinocyte undergoing apoptosis after 1,200j/m2 UVB radiation. (Photos courtesy of Walter Malorni, Istituto Superiore di Sanitá, Rome, Italy.) B. Typical DNA ladder, from cycloheximide-treated zebrafish cells. (Courtesy of Javier Negrón.)

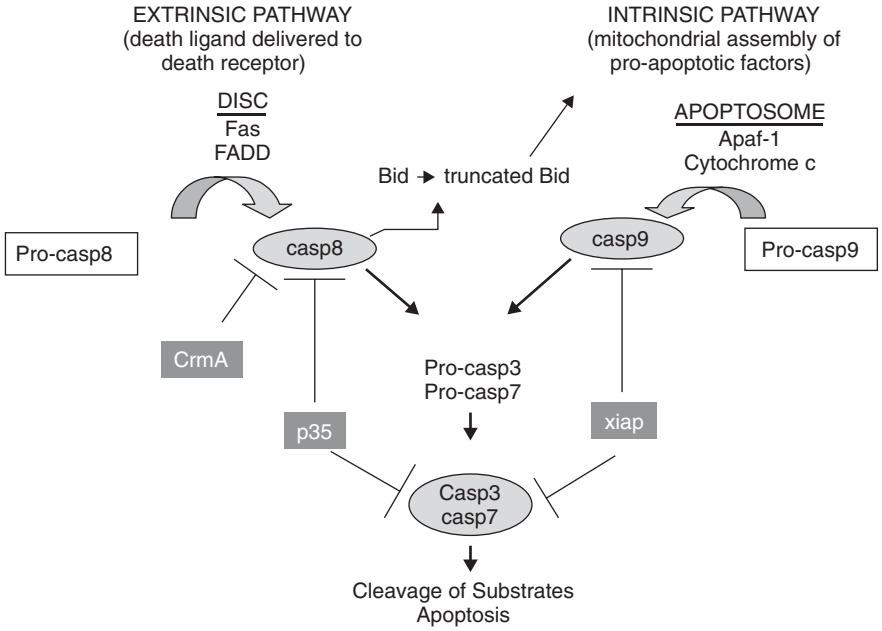


FIGURE 3. Control of caspase-dependent cell death in mammals. (After Salvesen, 2002.)

## Caspase-dependent Cell Death (initiated by mitochondrial changes)

Cell membrane signaling is typical for cell types, especially in the immune system, in which populations are rapidly changed. Thus, the Fas-FasL signaling system is extremely important in the embryonic establishment of lymphocyte types and immune tolerance as well as in the downregulation of T-lymphocytes during the resolution of an infection, and in several other situations (**Brás, García-Domingo, and Martínez-A**). However, often the decision to commit suicide depends on more internal considerations such as the health of the cell. This health is often evaluated by the status of mitochondria. Under various circumstances, in a series of related steps, mitochondria become permeable to several molecules, they depolarize, and they leak cytochrome c into the cytoplasm. The cytoplasmic cytochrome c displaces apoptosis-inhibiting factor (AIF) from pro-caspase 9, allowing its cleavage to the active form. Either active caspase 8 or active caspase 9 can activate caspase 3. The sensitivity of the mitochondria to various insults and stimuli can be adjusted by proteins that can be recruited to the mitochondrial membrane and either stabilize (bcl-2) or destabilize (bax) the mitochondria (Fig. 4). Mitochondria of course handle very dangerous materials—electrons

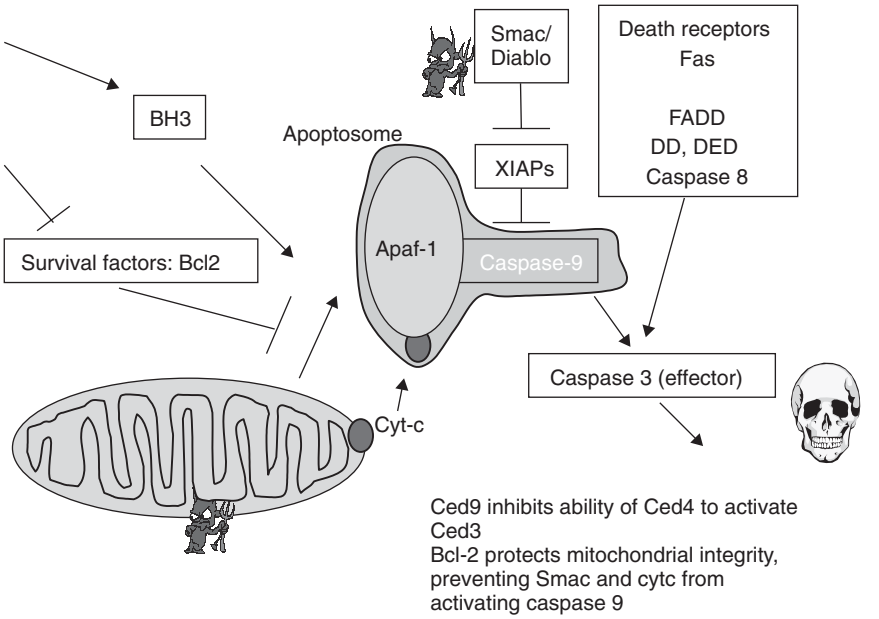


FIGURE 4. Mitochondrial and receptor-mediated caspase-dependent cell death in mammals.

transported one at a time through the cytochromes—and the possibility that reactive oxygen species will do damage increases with the disruption of metabolism. **Orrenius and Zhivotovsky** analyze the impact of this important factor, whereas **Vaux** discusses mitochondrial defenses, including regulation of the bcl-2 family, to contain the possible damage.

The resolution of apoptosis is an important characteristic, in that (with the exception of massively toxic insult) apoptotic fragments are phagocytosed by phagocytes or neighboring cells. As **Birge** outlines, phagocytosis is interesting on many accounts. There is evidence from *Caenorhabditis* that a phagocyte may play an active role in convincing a teetering cell to commit to apoptosis, and one of the remarkable features of apoptosis is the ability of the apoptotic cell to block an inflammatory response by the phagocytosing cell. The interplay between host and virus in controlling cell fate is complex, provocative, and revealing (**Chen, Fannjiang, and Hardwick**).

## Caspase-independent Cell Death

Caspases generate all the characteristics of apoptosis, including rounding and condensation of the cytoplasm (digestion of cytoskeletal proteins), condensation and margination of chromatin (digestion of chromosomal pro-

teins), internucleosomal degradation of DNA (activation of endonucleases), and exteriorization of phosphatidylserine (mechanism unclear). However, other physiological cell deaths can take place in the apparent absence of caspase activation, sometimes still displaying apoptotic morphology (Leist and Jäättelä, 2001a, b). In mutants that have lost caspase activity, cells may die by autophagic or other means. One argument is often misunderstood, but should be clear: If a cell has been severely compromised, or is deprived of its required growth factors, it will die. If it still has access to caspases, it will undergo apoptosis. If caspase activity is eliminated (by caspase inhibitors, mutation, or other means) the cell will still die, though perhaps not by apoptosis. Thus, the hope of preventing cell deaths by the inhibition of caspases has often proved disappointing.

It is also a bit tricky to make a clinical assessment of the impact of apoptosis in disease. The rate of mitosis can ultimately be traced by experimental labeling of newly synthesized DNA, but many estimates of the period during which an apoptotic cell is identifiable—based primarily on lymphocytes or thymocytes in vitro—fall in the range of 20 minutes to 1 hour, after which there is no sign that the death has occurred. Tidball and Albrecht (1998), using this kind of estimate, calculated that the entire liver could disappear in 1 month with only one apoptotic cell being seen per histological section. In the chapter by **Mani et al.**, two frequencies are given for different pathological situations in the myocardium: 0.25% of cells in a human, but 23 cells/100,000 (0.023%) in a mouse. If we assume a reasonable existence for an apoptotic thymocyte of 2.4 hours, the entire heart could disappear in 40 days to 4,000 days (the latter in a mouse, which lives approximately 720 days). However, one effort to establish a time for myocytes indicated an existence of perhaps 14 hours (Suzuki et al., 2001). As **Mani et al.** point out, the time it takes different cell types to undergo apoptosis can vary markedly over an order of magnitude; it is difficult to estimate apoptotic rates in chronic disease situations when the rate of cell loss is continuous and very low level; the rate of loss in these situations may not be a pure exponential but may change over time; one needs to know the duration of apoptosis in a given cell type to determine the impact of a given apoptotic rate on the loss of cells in that tissue; and because of these uncertainties, the only way to evaluate the importance of apoptosis to a given disease process is to inhibit and/or accelerate the apoptosis and see what it does to the disease process. In addition to two obvious unknowns (we do not know whether apoptosis is linear, asymptotic, or irregular with time, and we are assuming no mitotic replacement of cells—likely in the heart but not elsewhere), we have no meaningful estimate of the time of persistence of apoptotic cells. Thus, our quantitation of apoptosis in pathology is only very approximate. (We thank Richard Kitsis for the further elaboration of these points.)

## Autophagic Cell Death

The first description of lysosomes, and indeed the name, included the assumption that the rupture of lysosomes was the common form of cell death. This conclusion was apparently valid for the specific situation of carbon tetrachloride toxicity in liver, in which the $CCl_4$ could dissolve lysosomal and cell membranes, but it proved to be a simplistic interpretation of most other cell deaths. Nevertheless, the discovery launched a wide-ranging evaluation of lysosomal activity in cell death, an active field through the 1970s. These studies led to the conclusion that, in developmental cell deaths, the pool of lysosomal enzymes may be expanded as cell death is activated (Lockshin, 1969a; Lockshin and Williams, 1965d) or merely "activated" (formation of autophagic vacuoles rather than primary lysosomes—Helminen and Ericsson, 1970). As interpreted today, the lysosomes detected are either the lysosomes of phagocytic cells, or there is an expansion of the lysosomal system driven primarily by the formation of autophagic vacuoles. As is explained in the chapter by **Bursch et al.**, autophagic vacuoles are most clearly seen in large, postmitotic, sedentary cells. In these cells, the primary consideration is the removal of large amounts of cytoplasm, and the destruction of DNA is not a high priority. Thus, the early activities are the lysosomal destruction of cytoplasm, with DNA degradation occurring very late or not at all, and generally these deaths are not caspase-driven (see next section). Much remains to be learned of autophagic cell death. For instance, autophagy is often seen in atrophying cells, in which cytoplasm is reduced and the cell may enter a quiescent state, but the nucleus and the cell survive. The turning point or threshold is not well understood, although it may follow a schematic as presented by **Tolkovsky, Bampton, and Goemans**. Also, we know very little about the mechanisms by which the membranes of the autophagic vacuoles are formed, how they encircle target organelles or regions of cytoplasm, or how target organelles, such as mitochondria, expose markers or signals that identify them as targets. Many of the components are being identified (Klionsky and Emr, 2000), but the transients and control mechanisms remain to be explored.

## Necrosis

For a metazoan it is always preferable to control the death of cells, to contain the escape of potentially destructive molecules such as proteases and inflammatory cytokinins as well as invasive organisms such as viruses. Apoptosis, described below, contains the dying cell and avoids inflammation. Viruses, on the other hand, typically attempt to avoid this effective virus-controlling route. Their goal is not to lose their host cell or, if this is not an option, to provoke lysis and an inflammatory response through which they can escape. Therefore, viruses often have apoptosis-blocking mechanisms.

If the cell is very sick and cannot undergo apoptosis, it follows the route of necrosis. Likewise, when a cell is suddenly confronted with a severe stress,

such as a sharp change in tonicity, ion concentration, or pH, of the extracellular medium; or if all energy resources are suddenly extinguished, as in an infarct; if an increase or decrease of temperature makes the maintenance of homeostasis impossible; or if the integrity of the cell or organelle membranes is compromised by a solvent or physical disrupter of a membrane, the cell will simply rupture. The typical sequence is that mitochondrial failure will allow entrance of $Ca^{++}$ into mitochondria, swelling and rupture of mitochondria, loss of ion pumps, followed by loss of osmotic control of the cell, osmotic swelling and lysis of the cell, invasion of macrophages and inflammation, and removal of the debris. In electron microscope images, nucleoplasm and cytoplasm show disorganized precipitation of proteins, and there is no evidence of any active response of the cell to any stage of this disintegration (Fig. 5). The process is not stepwise and may follow different sequences.

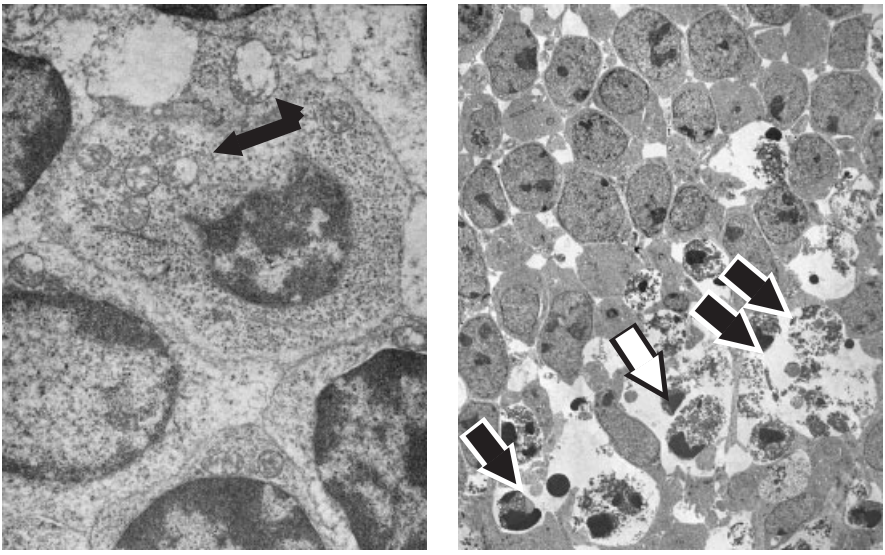

FIGURE 5. Necrosis. The figures illustrate swollen mitochondria (large arrow, upper left), some with tiny dense aggregates from an ischemic proximal convoluted tubule cell, and, following reflow, flocculent precipitations (larger arrow, lower left) and occasional calcifications. Later, the cells will show heavy vacuoliztion and ruptured organelles. (From Trump, Berezesky, and Osornio-Vargas (1981) with permission.) C. Apoptosis and necrosis in a mouse embryo treated with cyclophosphamide. In this instance, some cells undergo apoptosis (white-bordered black arrow); some appear to begin apoptosis, judging from the configuration of the nuclei (black-bordered white arrow); and some cells are frankly necrotic (double arrow). The necrosis almost certainly derives from the failure of overwhelmed phagocytes to remove the large number of apoptotic cells. (From the research of Daniela Quaglino, University of Modena, and Zahra Zakeri, Queens College of CUNY, with permission.)

## Other Forms of Cell Death

There are many other forms of cell death that show varying relationship to those best known. For instance, osteoblasts die as bones cease growing. Phagocytes cannot reach these cells and, although the death is assuredly programmed, ultimately the dying phagocyte looks necrotic. Keratinocytes eventually die by differentiating, in which the cytoplasm becomes completely occluded with insoluble proteins (mostly keratin). In this death the nuclei are destroyed, but, as tested by UV irradiation, the differentiation death is distinct from a true apoptotic response (Mammone et al., 2000). Similarly, the expulsion of organelles and eventual death of red blood cells share some characteristics of apoptosis (Bratosin et al., 2001). Differentiation of the lens involves the loss of nuclei from lens fiber cells. This loss, and the subsequent destruction of the DNA, involve the activation of a specific DNase and an apoptosislike destruction, although the lens fiber cells themselves persist and remain alive (Counis et al., 1998). Finally, the senescence of fibroblasts and other cells in culture is technically a differentiation rather than an apoptosis (**Warner**). Younger fibroblasts can undergo apoptosis, but the senescence is a form of differentiation, in which the cells become flattened squamous cells. They can persist for months in a postmitotic state, and their eventual death is not apoptotic (**Zakeri**). All these deaths are interesting in themselves, but remain beyond the scope of a book of reasonable size.

## History of Research on Cell Death

The development of the histological techniques of embedding, sectioning, and staining led to a flurry of observations, beginning in the late nineteenth century of cell activities of all types (Clarke and Clarke, 1996). These included, as a matter of course, many observations of cell death. Most of these were expected, as in insect and amphibian metamorphosis, and European scientists tried to make sense of the collapse of massive tissues. "Liquefaction" of tissues, invasion of phagocytes, vacuolization of cells, and nuclear shrinkage and swelling were noted. Organelles were not yet known and were not described. Several perceptive authors recognized similar configurations in what were otherwise considered to be healthy tissues and, coupled with the growing recognition of mitosis, the concept of cell turnover was born. Each of these deaths in adult tissues was usually considered to be unfortunate and unplanned. The extensive cell deaths in embryos were not yet known and, until Glücksmann's categorizations beginning in the 1950s (see Table 1), cell death was not considered to be a normal process except in metamorphosis (Glücksmann, 1951, 1965).

Glücksmann's compendiums are widely cited but often misunderstood. He clearly recognized that most embryonic deaths were not simple lysis. To quote him directly:

TABLE 1. Glücksmann's Classifications

| Stage/Localization | Number of examples |
|---|---|
| Embryogenesis | 7 |
| Organogenesis | 8 |
| Sense organs: eye, ear, nose | 13 |
| Epidermis, transient ectoderm | 3 |
| Digestive tract | 8 |
| Respiratory tract | 3 |
| Urogenital tract | 7 |
| Vascular system | 3 |
| Locomotion: notochord, somites, chondro- and osteogenesis, morphogenesis of muscles and skeleton | 21 |

| Types of degenerations | Subtypes |
|---|---|
| Morphogenetic | Changes in form of organs |
| | Ingrowth of tissue |
| | Union or detachment of parts |
| | Formation of lumina in organs |
| Histiogenetic | Differentiation of tissues and organs |
| | Formation of matrix and fibers |
| | Organ development |
| Phylogenetic | Vestigial organs |
| | Regression of larval organs |

> *Interference with the blood supply . . . causes a process of necrosis which is characterized by the loss of staining power of the nucleus, i.e., predominantly a process of autolysis leading to karyolysis and cytolysis. . . . The form of cellular death most frequently encountered in normal vertebrate embryos is characterized by a number of nuclear changes: (1) the initial stage, chromatopycnosis, consists in the separation of the chromatic from the non-chromatic material of the nucleus and the precipitation and coalescence of the former into larger granules and finally into a single mass. The non-chromatic material seems to liquefy and to form confluent vacuoles. (2) These nuclear changes result in the appearance of a single chromatic mass sitting as a cap on the vacuole formed by the non-chromatic material. This stage is described as hyperchromatosis of the nuclear membrane. Both the nucleus and the cytoplasm, which becomes liquefied or undergoes a fatty change, shrink by the loss of fluid. (3) After gradual shrinkage a mere chromatic granule persists and is surrounded by a liquefied or fatty zone. The granule loses its affinity for nuclear stains, becomes Feulgen-negative, breaks up and disappears: this is chromatolysis.*
>
> *(Glücksmann, 1951, pp. 60–65)*

Given the limitations of the time, this seems to be a fairly clear description of apoptosis, although without the final phagocytosis of the apoptotic frag-

ments. He further classified cell deaths based on their apparent "purpose"—morphogenetic, histiogenetic, or phylogenetic (Table 1)—but he did not make any statements concerning mechanism. His great contribution was to establish cell death as a normal biological process, occurring as a normal consequence of development and homeostasis, thus paving the way for its investigation.

Glücksmann's papers represent the culmination and end of the descriptive phase. By emphasizing the normality of cell death in embryonic development, he encouraged embryologists to investigate cell death as a developmental process. Several laboratories took different approaches to the study. Entomologists as well as those interested in amphibian metamorphosis were interested in endocrine and neural controls of metamorphic cell death (Kuwana, 1936; Finlayson, 1956). Dame Honor Fell understood the importance of cell death in the differentiation of cells in organ culture (Fell and Canti, 1934). Levi-Montalcini and Hamburger recognized the dependence of sympathetic and sensory neurons on peripheral tissues, although they quickly focused on the isolation and identification of nerve growth factor rather than the mechanism of death (Hamburger and Levi-Montalcini, 1949; Levi-Montalcini, 1987). Saunders identified specific regions of reproducible cell death in chick embryos (Saunders, 1966; Saunders and Fallon, 1966). Finally, John Kerr, an Australian pathologist, began to recognize many commonalities in widely divergent deaths. These experiments inaugurated the experimental phase and led to the related concepts of programmed cell death and apoptosis (Kerr, 1965).

From the information that the death of certain cells in embryos is predictable, one can infer that, like any developmental characteristic, it is in some manner encoded in genes and therefore must be controlled by a developmental program. In chick embryos, the digits and axes of limbs are sculpted in part by the death of specific cells. Saunders explanted such cells, from the "posterior necrotic zone," of the limb bud to tissue culture and found that they died on schedule. However, these were not simply dying cells taking their time to manifest the morphology of death, since transplanting them to another region allowed them to heal in and survive (Fig. 6). Thus, the death sentence was reversible, and although, in Saunders' words, "the death clock is ticking" (Saunders, 1966), the death of the cells could be reversed.

In a similar vein, Lockshin and Williams established that several markers of the impending death of insect muscles (larval muscles of metamorphosing insects) could be identified while the death remained reversible. These experiments introduced the term "programmed cell death," meaning that a specified physiological sequence in otherwise healthy cells led to their death, and that the death was not imposed by a toxic or inhospitable environment. Among the markers of impending cell death was the activation of the lysosomal system (Table 2). These, then recently discovered, organelles were at

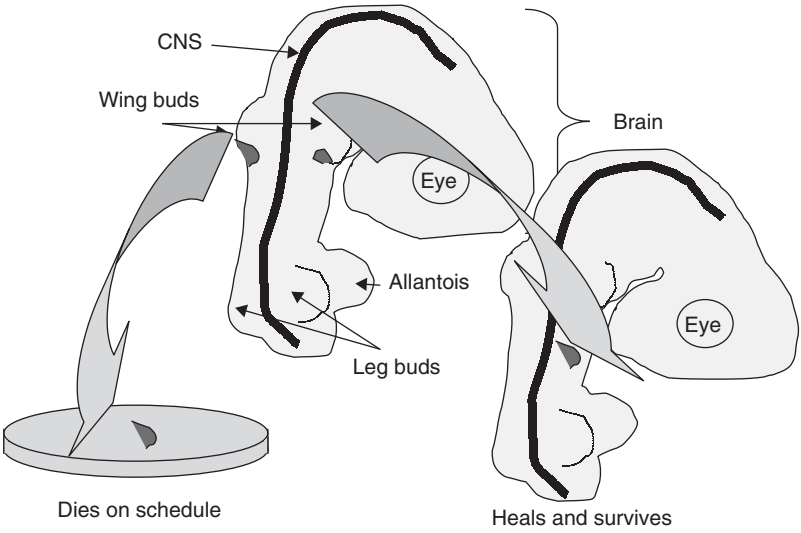


FIGURE 6. Saunders's experiments. In these experiments, Saunders removed the posterior necrotic zone from the forelimb of a chick as soon as the region was anatomically identifiable, but many hours before the cells were scheduled to die. When he explanted the tissue to a Petri dish and cultured it, the cells died on schedule, indicating that at the time he excised them, they already possessed the information that would lead to their deaths. If instead of explanting the tissue, he transplanted it to the back of a second host chick, the tissue healed into the epidermis, and the cells did not die, but differentiated in concordance with their host environment, demonstrating that the information to die was not a mortal but slowly developing restriction to the cells.

the time considered to be the potential effectors of cell death, a hypothesis widely discussed at the time, later forgotten, and of renewed interest at the beginning of the twenty-first century.

The hypothesis of programming carried the inference of an ultimate genetic origin, but it took the clarity of *Caenorhabditis* genetics to define what this meant. Briefly, in a series of brilliant experiments, Horvitz, Sulston, and colleagues showed that a limited number of genes controlled virtually all embryonic cell deaths in this worm (Ellis and Horvitz, 1986; Horvitz, Shaham, and Hengartner, 1994) (Fig. 7). The establishment of the genetics of cell death gave full meaning to the hypothesis of programming and quickly led to recognition that the genetics of cell death was conserved even in mammals and to the recognition that a major effector of cell death was a specific protease (Figs. 7 and 8).

Simultaneously, Kerr and colleagues realized that many types of cells in pathological and nonpathological situations tended to shrink and bleb; their cytoplasm became dense, their organelles intact; and the chromatin of their

TABLE 2. Why Cell Death Is Programmed (metamorphosing insect muscles)

| Stage of development | Event |
|---|---|
| Day 0 | Pupal-adult metamorphosis begins. |
| Days 0–4 | Ecdysone in absence of juvenile hormone sets adult development; muscles also acquire signal to die; injection of juvenile hormone blocks this signal. |
| Day 17 | Beginning appearance of lysosomes; slight decrease in muscle mass. |
| Day 21 (hours 0–2) | *Neural:* Ecdysis (emergence of adult); high neural activity, then spontaneous quiescence of CNS activity. |
| | Pharmacological or electrical maintenance of neural activity prevents muscle loss. |
| | Pharmacological blockage of neural activity causes slightly premature death of muscles. |
| | *Genetic:* Slight burst of synthesis of specific proteins; rapid drop of synthesis of others. |
| | Cycloheximide and actinomycin D prevent degeneration of muscles. |
| Day 21 (hours 2–5) | Rapid increase in measurable (free) lysosomal proteolytic activity. |
| | Increase in rate of loss of muscle protein. |
| | End of ability of cycloheximide to block death. |
| Day 21 (hours 6–10) | Increase in autophagic vacuoles, targeting heavily mitochondria. |
| | Continued loss of muscle protein. |
| Day 21 (hours 10–15) | Loss of membrane resistance, depolarization of muscle membranes, muscles enter flaccid paralysis. |
| | Expansion of autophagy; dissolution (in cytoplasm) of myofilaments. |
| | Erosion of extracellular matrix (sarcolemma). |
| Day 21 (hours 15–24) | Condensation of chromatin to heterochromatin; phagocytic consumption of remaining tissues. |

*Source* From Lockshin and Williams (1964, 1965a–d) and Lockshin (1969b).

nuclei condensed along the periphery of the nucleus. This "shrinkage necrosis" was difficult to explain. A cell that for any reason can no longer succeed with oxidative phosphorylation follows a specific, easily comprehensible sequence: It generates its remaining energy through glycolysis. Before the cell becomes very acid, lactate ion does not easily escape, osmotically drawing in water and swelling the cell until it lyses. This is necrosis as described above. It was much harder to explain shrinkage necrosis, which in a now famous paper was rechristened "apoptosis" (Kerr, Wyllie, and Currie, 1972). The term apoptosis was a morphological description begging

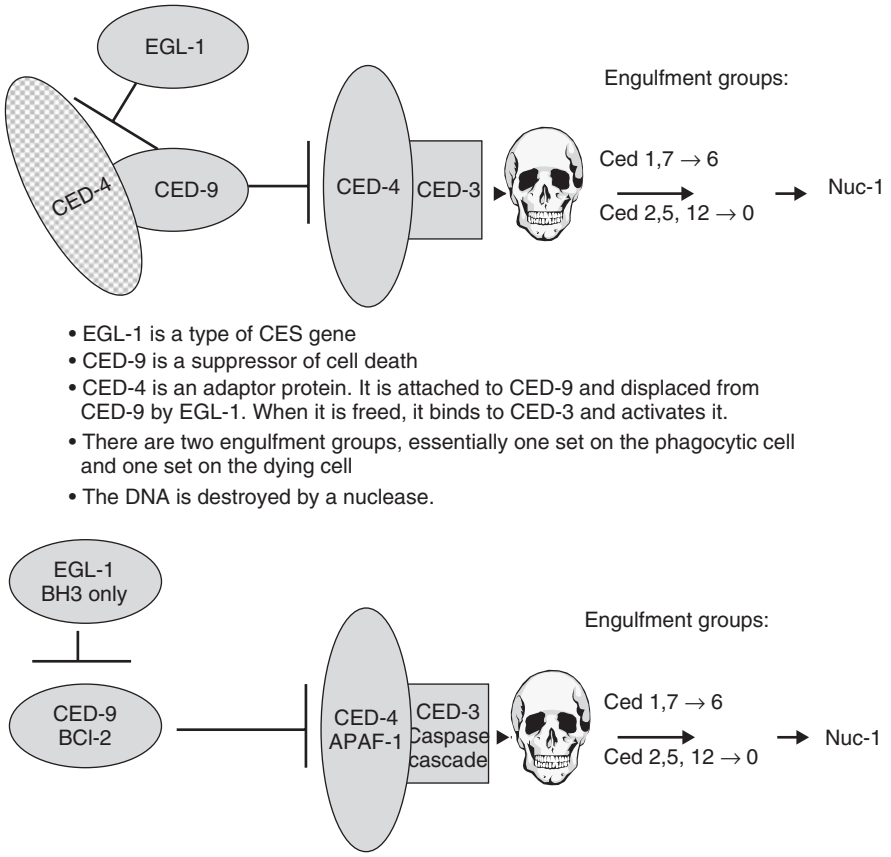


FIGURE 7. Upper: Control of cell death in *Caenorhabditis elegans*. Lower: The parallel structure of cell death in mammals. This parallelism is illustrated in more detail as a flowchart in Figure 8.

an explanation. For a cell to shrink, it must either contract (but the cytoskeleton cannot generate sufficient force to extrude water) or lose solute (Lockshin and Beaulaton, 1981). And what was one to make of the collapsed chromatin?

The shrinkage that occurs during apoptosis now appears to result from the loss of $K^+$ from the cell (Bortner and Cidlowski, 2002; Razik and Cidlowski, 2002). The collapse of the chromatin appears to derive from the degradation of the DNA (Arends, Morris, and Wyllie, 1990), itself deriving from the caspase-initiated activation of a protease (Weaver et al., 1993).

The flowering of the *Caenorhabditis* story, both the universality of its genetics and the discovery of the caspase family of proteases, coincided with the appearance of cell death (apoptosis) on the clinical horizon. Several discoveries led to the medical interest, although some in particular caught atten-

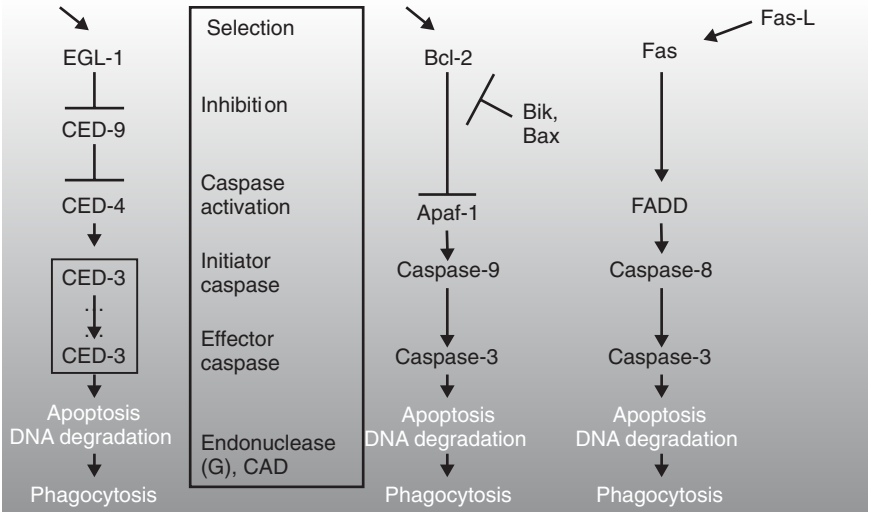


FIGURE 8. Overall scheme of caspase-dependent cell deaths in *Caenorhabditis* and mammals.

tion: (1) B-cell lymphoma arose from a chromosomal translocation that constitutively activated bcl-2, protecting lymphocytes from death and producing a tumor by lack of cell death rather than excess mitosis—with bcl-2 being recognized as the first antiapoptosis gene. (2) The interaction of a soluble or cell-surface molecule, Fas ligand, with the cell-surface molecule Fas or Apo-1 could spectacularly destroy certain tumor (and many other) cells, thus suggesting that the active killing of tumor cells was important in both tumor prognosis and immune dysfunction. (3) Jean-Claude Ameisen broached the idea that the catastrophic loss of bystander CD4+ cells in AIDS could result from an improper management of apoptosis (Ameisen and Capron, 1991). Clinicians on several fronts became very interested, and the accessibility of some reasonably straightforward assays for the DNA damage marking apoptosis (electrophoresis of DNA, FACS analysis, TUNEL staining (**Zakeri**) led to the upsurge of interest that we now see. Several authors have given their versions of the history of the field (Clarke and Clarke, 1996; Lockshin, 1997; Lockshin and Zakeri, 2001; Vaux, 2002).

## Evolution of Apoptosis

Since apoptosis is so carefully conserved among all metazoans, the question naturally arises as to its origin. As Ameisen has asked, either apoptosis was created out of whole cloth or it arose as a modification of a preexistent mech-

anism. The former is improbable. But what was the preexistent mechanism? Plants manifest many instances of programmed cell death, but they do not have caspases, and their cell deaths are only equivocally apoptotic (**Mittler and Cheung**). *Dictyostelium* stalk cells undergo programmed cell death, but again not an apoptotic one (**Levraud et al.**). For multicellular organisms, there is a clear advantage in being able to control the death of cells (**Chen, Fannjiang, and Hardwick**). All organisms appear to exploit lysosomal systems already available for cytoplasmic reduction, but metazoans evolved true apoptotic cell death.

Proteases distantly related to caspases exist in all cells and perform various functions related to housekeeping and mitosis. Ameisen, identifying apoptosislike phenomena in protozoans such as *Tetrahymena*, suggests two means of creating apoptosis: Quorum sensing, known in bacteria, allows a population of individual cells to respond individually to the condition of a population. Thus, if each individual secretes a small amount of a signaling molecule, the individual cell can sense the population density. Such a mechanism regulates the rate of cell division in the liver of a mammal. Populations can therefore be selected on the basis of their ability to maintain population density within sustainable limits, and the capacity for cell death can become a selective advantage. Second, a host can become addicted to a parasite in the following manner: The parasite introduces into its host a toxin but induces a host antidote (often a protease). However, the antidote has a half-life substantially shorter than the toxin. Any host that loses the parasite is doomed because the toxin will outlast the antidote. Any parasite that can maintain such an "addiction cassette" of toxin and inducer will condemn its host to bear it forever. Such arrangements are well known in microorganisms and are sustained according to the Red Queen hypothesis (Ameisen, 1996, 1998, 1999). As in the Alice in Wonderland story, in which the Red Queen engages Alice in a race that returns to the starting line, the establishment of an addiction cassette engages a perpetual race in which the host evolves to escape the parasite, selecting for parasites that do not escape, and the coevolution goes faster and faster while going nowhere. Interestingly, one of the most effective toxins (inducers of apoptosis) is cytoplasmic cytochrome c. When cytochrome c escapes from mitochondria, it activates a proteolytic cascade terminating in apoptosis. Given the presumptive origin of mitochondria from parasitic or commensal bacteria, the similarity to addiction cassettes is striking. Sequence analysis leads to a conclusion consistent with this hypothesis. Koonin and Aravind, who have attempted to trace the origins of caspases and other apoptosis-related genes (Aravind, Dixit, and Koonin, 2001; Koonin and Aravind, 2002), find that "homologs of apoptotic proteins are particularly abundant and diverse in bacteria that undergo complex development, such as Actinomycetes, Cyanobacteria and α-proteobacteria, the latter being progenitors of the mitochondria" (p. 394). The apoptosis-related proteins most commonly form multidomain proteins.

Multidomain construction is most commonly associated with the signal transduction and regulation of gene expression. The genes for the apoptosis-related proteins indicate considerable fusion with other genes, suggesting that the endosymbiosis of bacteria brought to eukaryotes several important apoptotic effectors. These effectors were subsequently modified by horizontal gene transfer, so that their adaptor and promoter regions were selected for eukaryotic use. Some of these rearrangements included the transfer of the genes to nuclear rather than mitochondrial control.

## The Future

It is always dangerous to predict the future. RAL remembers as an undergraduate getting very high marks for two papers that completely missed what were to become the hottest stories of the time (clonal selection theory and the function of the thymus) and later realizing that even his professors had failed to recognize the breakthroughs. Nevertheless, we are approaching the threshold of clinical applications for apoptosis, and it is useful to see where we stand. In the last part of the book we address the role of apoptosis in specific organ systems: the immune system (**Brás, García-Domingo, and Martínez-A**), gastrointestinal tract (**Wilson and Potten**), and nervous system (**Tolkovsky, Bampton, and Goemans**), as well as the role of apoptosis in aging (**Warner**). We follow with chapters on apoptosis in the specific disease states of viral infection (**Chen, Fannjiang, and Hardwick**), cancer (**Fulda and Debatin**), and myocardial infarct (**Mani et al.**). To a very large extent, any therapies based on apoptosis will depend on highly accurate targeting, a sort of smart missile technology to destroy or protect individual cells. This is why the application of proteomics to apoptosis theory (**Saelens et al.**) will prove so important. Nevertheless, since with the obvious exception of bcl-2 mutations (B-cell lymphoma) and p53 mutations (many cancers), the machinery of apoptosis is intact in a pathological situation, but the threshold of response is altered, in the long run we will need to understand much better the more subtle aspects of metabolic history that set those thresholds.

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SECTION

II

# Biological Role of Cell Death in Development and Homeostasis

CHAPTER

2

# CELL DEATH: SHAPING AN EMBRYO

ZAHRA ZAKERI AND RICHARD A. LOCKSHIN

## DEVELOPMENT: THE BASIS FOR THE CONCEPT OF CELL DEATH

Developing systems have always been at the forefront of studies of cell death. Classical naturalists recognized the disappearance of larval structures in metamorphosing insects and amphibia. Vesalius had recognized the transience of the ductus venosus in the sixteenth century, and Harvey had observed the remodeling of the embryonic heart in the seventeenth century. However, a true recognition of the significance of cell death required the discovery of microscopic lenses by van Leeuwenhoek in the late seventeenth century, followed by the development of cell theory in the mid-nineteenth century, and the development of sectioning and staining technology during the same period. Observation of cell death as a naturally occurring phenomenon followed remarkably quickly, as embryologists noticed the presence of cells with unusual morphology. Vogt in 1842 noted the disappearance of the notochord of the midwife toad during metamorphosis (cited in Clarke and Clarke, 1996; see also Ranganath and Nagashree, 2001). Embryologists were comfortable with the possibility of transient organs. However, growth of a fascination with mitosis took place in the context of an understanding that mitosis (the active, constructive activity) was used to balance the acci-

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dental and unfortunate unpredictable loss of cells. By the 1950s some change in attitude became apparent. Hamburger and Levi-Montalcini, in the quest that ultimately led to the discovery of nerve growth factor, recognized that many neurons were lost during the course of normal neurogenesis (Hamburger and Levi-Montalcini, 1949), and Saunders, noting specific patches of dead cells in the developing limbs of chicks, began a quest that led to the recognition of controlled cell death in the embryo (Saunders, 1948). Lockshin and Williams, working with insect metamorphosis, extended the concept and described these deaths as "programmed" (Lockshin and Williams, 1965a, b, c). By the 1970s Kerr, Wyllie, and Currie had established the similarities of many cell deaths in many organisms and had amalgamated these descriptions under the rubric "apoptosis" (Kerr, Wyllie, and Currie, 1972). By the 1980s the existence of specific cell death genes was demonstrated for the nematode worm *Caenorhabditis*, and between 1989 and 1991 cell death was identified as an important component in lymphomas, in AIDS, and in autoimmune disease and differentiation of the immunological system in general. The discovery of the clinical relevance of cell death launched the frenzy that we are now experiencing, including a revisiting of the role of cell death in developing systems.

## Naturally Occurring Cell Death in Embryos

We cannot possibly review all the developing systems in which cell death plays a role. We will therefore examine cell death in a few developing systems, with an emphasis on some of the methodology used. Some of the most valuable techniques for the study of apoptosis in maturing or mature immune systems or in cultured cells of immunological origin, such as flow cytometry, are rarely applicable to embryos. In embryos, the small number of cells and the importance of temporal and spatial distribution of cell death necessitate the use of techniques such as immunocytochemistry and in situ hybridization.

Early observations were essentially entirely morphological, leading to the recognition that patterns of death could be predicted and identified in most embryos, and therefore to the conclusion that cell death is as much a part of normal development as proliferation or differentiation (Glücksmann, 1951; Saunders, 1966; Hinchliffe, 1981; Coucouvanis, Martin, and Nadeau, 1995; Chanoine and Hardy, 2003). Glücksmann also importantly recognized that cell death continued throughout life, including cell turnover and even pathology, and did not distinguish any significant distinction in the mechanisms of these deaths, thus presaging a conflation of all physiological cell deaths.

Embryonic development is dynamic and extremely well coordinated, making an embryo ideal for the study of the control of cell death. The promi-

nence of cell death in development was the source of most of the theoretical bases of cell death research in developmental biology, including programming, identification of specific cell death genes, and discovery of the caspases. However, in a developing embryo, a dying cell is often surrounded by many dividing and/or differentiating cells, making its identification difficult. Also, the onset of cell death is usually not synchronous, making any study of the kinetics of cell death more difficult.

In embryos, cross-talk among cells (mutual induction loops) and the ever-changing microenvironment of cells are extremely important in establishing the level of determination and fate of cells, including both cell division and cell death. As is described in the introduction to this book, Saunders's brilliant transplantation studies established the importance of the microenvironment (Saunders, 1966; Saunders and Fallon, 1966). Local fields establish patterns of cell death, as is clearly seen where mutations producing defects in patterns lead to malformations (Zakeri, Quaglino, and Ahuja, 1994; Singh, James, and Zakeri, 1997). We now know that most cells die in the context of their environment (Lockshin, Osborne, and Zakeri, 2000) or, as Raff (1992) described it, there is a "social control" of apoptosis. However, one must keep in mind that in embryonic development the social control signals rapidly undergo changes due to the rapid change in the social milieu.

Embryonic cell death occurs throughout the animal kingdom. Neuronal, muscle, epithelial, intestinal, and gonadal cells undergo cell death in the developing nematode, *C. elegans* (Sulston and Horvitz, 1977; Ellis and Horvitz, 1986; Hengartner and Horvitz, 1994). Insect metamorphosis is accompanied by the death of larval muscles (Lockshin and Beaulaton, 1981; Haas et al., 1995; Schwartz and Truman, 1982); nerves (Truman, 1984; Fahrbach, Choi, and Truman, 1994); and glands (Jochová, Zakeri, and Lockshin, 1997). In the higher insects, almost all larval tissues are destroyed at metamorphosis, and in anurans the tail and gills of the tadpole are wholly destroyed and in many other tissues there is substantial apoptosis during remodeling (Weber, 1969; Fox, 1973). In tadpoles, regression of the tail and destruction of the gills are attributed to tightly controlled cell death (Jeffery, 2002). In *Xenopus*, cell death destroys all the primary myotomal myofibers, which are replaced progressively by secondary "adult" multinucleated myofibers in the construction of muscles during development (Chanoine and Hardy, 2003). In *Drosophila*, cell death is induced in larval midgut by the steroid hormone ecdysone (Lee, Cooksey, and Baehrecke, 2002).

In mammalian embryos, cell death starts as early as two cell stage as the polar bodies die and continue in the embryo proper as soon as the inner cell mass is formed (Fig. 1e; Zakeri et al. in preparation; also Hardy, Handyside, and Winston, 1989; for review, see Spanos et al., 2002). Apoptosis has been seen in fetal membranes where changes such as the condensation of chromatin along the periphery of the nucleus and nuclear shrinkage have also been seen in amniotic epithelium and chorionic trophoblast cells (Wiley

et al., 1992; Nijhawan, Honarpour, and Wang, 2000). Cell death can also be found in the forming placenta as the embryo implants into the wall of the uterus (Zakeri, unpublished). Later, cell death shapes many organs and removes interdigital webs during limb development (Saunders and Fallon, 1966; Hinchliffe, 1981; Hurle et al., 1996). The specific spatial and temporal patterns of cell death are, as expected, species-dependent. During limb formation, cell death is first observed in the anterior and posterior marginal zones (AMZ and PMZ) of the developing limb bud. In later stages, there is massive cell death in almost all the interdigital mesenchymal tissue (IMT) located between the chondrifying digits (Hurle et al., 1996). The fate of the IMT is determined by the overlying ectoderm as well as the presence of adjacent digits. Cell death in the developing AER is thought to limit the size of the AER and subridge and therefore prevent polydactyly (Hurle et al., 1996). These patterns of death free the digits of birds (Saunders and Fallon, 1966) and mammals (Ballard and Holt, 1968).

The patterns and connections of the developing nervous system depend heavily on cell death (Hamburger and Oppenheim, 1967). During vertebrate development, more nerve cells are produced than are needed, and 20 to 80% of the neurons that are born die (Gordon, 1995; Clarke et al., 1998). Fetal neurons compete for limited amounts of nerve growth factor (NGF), a survival factor produced by other cells, including neurons. Thus, apoptosis induced by growth factor withdrawal adjusts the numbers of developing neurons to the number of target cells (Jacobson, Weil, and Raff, 1997; Reis et al., 2002; Vyas et al., 2002). More than 80% of ganglion cells in the cat retina die shortly after birth (Barres and Raff, 1999). In secondary palate formation, the fusion of two opposing palatal shelves is accomplished by cell death in the midline epithelium (Shapiro and Sweney, 1969). Likewise, immunological tolerance is defined by the destruction of cells bearing potential antiself immunoglobulins and "neglected" cells bearing nonfunctional immunoglobulins also die (Osborne, 1998; Gercel-Taylor et al., 2002). Positive and negative selection of thymocytes in embryonic and early postnatal mammals leads to a vast repertory of immunocompetent cells coupled with self-tolerance. The achievement of this grand design derives from a complex series of interactions including steroids, T-cell activations, and the relative expression of Fas and FasL (Osborne, 1998). The fact that lpr/lpr mice (which express little or no functional Fas) and gld/gld mice (which express little or no functional FasL) develop lupuslike syndromes indicates that this ligand–receptor interaction is important in negative selection in the thymus.

In almost all developing organs at one time or another, one can find dying cells that help to sculpt the organ. An example occurs in the lens, where apoptosis plays a major role during lens vesicle development. Here, apoptosis eliminates the cells between the surface ectoderm and the optic vesicle to help trigger invagination and facilitate separation from the ectoderm. Apoptosis also aids in the bowing of the optic vesicle during lens

invagination (Mohamed and Amemiya, 2003). Apoptotic cell death helps shape the future inner ear structure, which starts from incubation day 5 in chick inner ear (Avallone et al., 2002). In cardiac morphogenesis, cell death is essential in generating the overall four-chambered architecture of the heart (Abdelwahid, Pelliniemi, and Jokinen, 2002). In rat skeletal muscle, cell death persists during the first three postnatal weeks, suggesting an indispensable role for cell death in the development of skeletal muscle (de Torres et al., 2002).

There are also many pseudodeaths or partial deaths in living organisms. In the developing lens, the nuclei of lens fiber cells undergo nuclear apoptosis, but the cytoplasm survives and the cells remain as normal functional cells after the 18th day of gestation of embryonic mice (Appleby and Modak, 1977; Wyllie, Kerr, and Currie, 1980; Gao et al., 1997; Nijhawan, Honarpour, and Wang, 2000; Gupta, Tsai, and Wynshaw-Boris, 2002; Wu et al., 2002). Likewise, mammalian red blood cells lose their nuclei, and epidermal keratinocytes ultimately keratinize and die.

Cell death also plays an important role in the development of the reproductive system as the decision is made for regression of the male (Wolffian) or female (Müllerian) duct systems to produce a male or female embryo (Dyche, 1979). In adults, cell death remains a factor in the maintenance, integrity, and function of the gonads. In the testis cell death is important for normal spermatogenesis and helps to regulate sperm output (Blanco-Rodríguez, 1998; Hikim and Swerdloff, 1999; Kierszenbaum, 2001). The maintenance of the different cell types results in the degeneration of up to 75% of potential spermatozoa (Huckins, 1978; Allan, Harmon, and Kerr, 1987). In human females, oocyte apoptosis results in the loss of more than 80% of the original germ cells just by birth and a major level of cell death is seen in atretic follicles (Tilly, 1998; Reynaud and Driancourt, 2000).

Plant fertilization, embryogenesis, and development all involve substantial cell death, and plants use cell death in response to environmental stimuli such as cold and attacks by bacteria and viruses. A major role for cell death in plants is the formation of the sexual organs, in essence much like the mammals (Pennell and Lamb, 2001). Although death in plant cells sometimes resembles apoptosis, there are also unique types of cell death seen in plants in which the vacuoles play a critical role (Fukuda, 2001; Mittler and Cheung, this volume). Botanists use similar methodologies to study cell death in plants.

## Genetic Control of Developmental Cell Death

The notion of genetic regulation of cell death was realized due to the existence of a number of mutant animals with aberrant patterns of cell death such as those seen in mutant mice strains with syndactyly and webbed limbs,

in which cell death appears to fail, and the mapping of the sequence of events in interdigital segregation led to the idea that cell death is under direct or indirect genetic control (Johnson, 1969; Hinchliffe and Thorogood, 1974; Ingham and Martinez-Arias, 1992; Zakeri, Quaglino, and Ahuja, 1994a).

Studies of *Caenorhabditis* were crucial to our understanding of the genetic regulation of apoptosis, in that the cell death mutants identified in *Caenorhabditis* controlled all embryonic or developmental cell deaths, leading to the now well-known sequence of bcl-2-like molecules normally inhibiting cell death, the activation of caspaselike molecules destroying the cells, and the existence of "ready for phagocytosis" signals (phosphatidylserine and other molecules) on the surface of apoptotic cells and their counterpart receptors on the phagocytes. A similar series of genes, the reaper-hid-grim group, was identified from their effects on embryonic cell deaths in *Drosophila*, although this group, unlike the ced (cell death) genes in *Caenorhabditis*, does not appear to be universal. Beyond these studies, incidental papers have detected cell death in preimplantation mammalian embryos, and an interesting study suggested that there existed in amphibian embryos an inhibitor of apoptosis that disappeared at the maternal-zygotic transition (Hensey and Gautier, 1997). Similar observations in zebrafish have been reinterpreted to suggest that the capacity to undergo apoptosis is acquired at the maternal-zygotic transition, thereby providing access to the critical determinants of the apoptosis machinery (Negrón and Lockshin, in preparation). Much can be learned about the function of a gene by manipulating its expression. This approach has been used to study the function of a number of cell-death-related genes. Since the knockout in many systems produces an identifiable phenotype in the developing embryo, we have learned much about the role of these genes in the developing embryos.

However, knockout of many genes influencing cell death results in no specific embryonic phenotype. For instance, p53 knockout mice, which are deficient in the ability of their precancerous cells to undergo apoptosis, present no abnormal phenotype of cell death in the embryo (Donehower et al., 1992), although they do develop spontaneous tumors (Jacks et al., 1994; Williams et al., 1994). This is a generic problem in using knockouts to determine function. Embryos frequently use partial redundancy to compensate for the loss of specific genes and thus drastically reduce the phenotypic effect of a knockout. This compensation includes cell death mechanisms. We and others have shown that cyclin-dependent kinase 5 (Cdk5) plays a role in cell death in both adult and embryonic tissues during development (Singh Ahuja, Zhu, and Zakeri, 1997; Zhu et al., 2002). However, cdk5−/− embryos are normal and present at the expected frequency (24%) as late as gestation day 16.5 (Ohshima et al., 1996). Furthermore, knockouts of the regulator of cdk5, p35−/− embryos develop normally, with no gross anatomical defects found in somatic organs or tissues (Chae et al., 1997). Bak−/− mice are developmentally normal and reproductively fit and do not develop any age-

related disorders (Lindsten et al., 2000). Knockouts of bcl-2 show minor abnormalities: At E14 in vivo, the number of trigeminal neurons undergoing apoptosis was significantly greater in bcl-2–/– embryos, and there were significantly fewer neurons in the trigeminal ganglia of bcl-2–/– embryos at E16 and E18 (Pinon, Middleton, and Davies, 1997).

Mice deficient in another antiapoptotic bcl-2 family member, bcl-$X_L$, die at embryonic day 13 with excess apoptosis in developing brain, spinal cord, and dorsal root ganglia (Motoyama et al., 1995). Among the cell death genes, caspases appear to be important in developmental cell deaths. Deletion of the key death effector caspase, caspase-3, in mice in most cases is lethal to the embryo. Caspase-3–/– mice are born at a frequency lower than expected by Mendelian genetics, are small, and die before 3 weeks. The brain development in caspase-3-deficient mice is profoundly affected, with the mice exhibiting a variety of hyperplasias and disorganized cell deployment in the cerebral cortex, the hippocampus, and the striatum. Furthermore, pyknotic clusters at sites of major morphogenetic change during normal brain development are not observed in the mutant embryos, suggesting decreased apoptosis in the absence of caspase-3 (Kuida et al., 1996). Deletion of caspase-9 yields a phenotype essentially identical to that of caspase-3 null mice (Hakem et al., 1998; Kuida et al., 1998). Mice deficient for Apaf-1 (apoptosis protease-activating factor 1, which activates caspase-9) have a strong phenotype similar to that of caspase-3 and -9 knockouts, revealing an apparently lower incidence of apoptotic cells in their hindbrains compared to wild-type mice. In addition, Apaf-1 knockout mice exhibit delayed interdigital mesenchymal cell death and alterations in the development of lens and retina (Colussi and Kumar, 1999). Mice carrying a null mutation in caspase-2 develop normally and do not show an overt phenotype. The most prominent feature of caspase-2-deficient mice, an inhibition of female germ cell death, results in knockout mice containing a significantly higher number of primordial follicles compared to the wild type (Bergeron, 1998). Null mutations in caspase-8 result in embryonic lethality in mice that have developmental abnormalities in cardiac tissue (thin ventricular myocardium) and hyperemia in the abdomen and other blood vessels with extensive erythrocytosis in the liver (Varfoloweev et al., 1998; Yeh et al., 1998). Thus, genetic inhibition of caspases has profound effects on development. However, the phenotype of the caspase-8 knockout suggests functions other than cell death for caspases. Furthermore, Oppenheim et al., examining further the caspase-9 knockout, concluded that the elimination of this initiator caspase did not prevent cell death; it only changed the morphology of the deaths (Oppenheim et al., 2001). Therefore, we have much to learn about the role of presumptively apoptosis-specific enzymes in embryos.

From these studies, it is apparent that cell death in the embryo is governed by gene action and that many of the cell-death-related genes have an effect early in the developing embryo, indicating both the importance of their

role in the correct implementation of cell death and the role of cell death in the correct formation of the embryo.

Abnormalities in development can stem from mutations or environmental factors that transiently affect the developing embryo. Production of transgenic mice often leads to abnormalities in the embryo as mentioned above, but many other factors can also lead to developmental abnormalities. A number of investigators working on how teratogens including thalidomide, ethanol, neuroactive drugs, and chemotherapeutic agents such as cyclophosphamide lead to abnormalities have found that a major effect is the deregulation of pathways of normal cell death in the developing embryo (Mirkes, 1985; Alles and Sulik, 1989; Zakeri, Quaglino, and Ahuja, 1994; Zakeri and Ahuja, 1997; Little and Mirkes, 2002; Zhu et al., 2002).

## Functional Needs of Cell Death During Development

Cell death has been classified into several categories based on function or evolution (Glücksmann, 1951; Ellis, Yuan, and Horvitz, 1991): Some cells perhaps serve a prior evolutionary purpose, but provide no current function and undergo a phylogenetic cell death (Fallon and Simandl, 1978). Morphogenetic cell death permits differentiation of a tissue to its final form (Glücksmann, 1951; Hinchliffe, 1981). Histogenic cell death modifies a tissue so that it acquires function, or enables the tissue to function differently from a similar tissue type (Glücksmann, 1951). For example, three different patterns of cell death occur in the somites of the developing embryo. These variations are dependent on the stage of the embryo and on the path that migrating neural crest cells take (Jeffs and Osmond, 1992; Coucouvanis, Martin, and Nadeau, 1995). Cell death may also occur in one sex as a means of differentiating sexually dimorphic traits. For example, sexual differentiation in vertebrates involves the hormonal control of cell death of reproductive structures such as the Müllerian and Wolffian ducts (Scheib, 1963). Numbers of developing neurons substantially exceed that required and therefore some are removed (Hamburger and Levi-Montalcini, 1949; Cowan et al., 1984; O'Leary, 1987). There is less demand on DNA-encoded information if the nervous system is wired by excess production and survival of the best-wired rather than plans for specific wiring of each neuron. Cells may be necessary at one stage of development and then no longer required, such as the tadpole tail during metamorphosis (Kerr, Harmon, and Searle, 1974; Lockshin, 1981). Some cells may be defective in shape or function such as lymphocytes that have either failed to produce functional antigen-specific receptors or produce autoantibodies, threatening an autoimmune attack on the organism (Cohen, 1991; Golstein, Ojcius, and Young, 1991; McCarthy, Smith, and Williams, 1992; Osborne, 1995). Therefore, cell death accounts for

the deletion of cells that takes place in normal tissues. Where it occurs pathologically, it may serve an adaptive or homeostatic role (Walker et al., 1988).

## Types of Cell Death

Our understanding of cell death was clarified by the definition of apoptosis by Kerr, Wyllie, and Currie (1972). Following a period of acrimony over a binary distinction (between apoptosis and necrosis) Schweichel and Merker (1973) attempted to classify cell death into at least three types, as defined by high-resolution cytology, in developing embryos. They described three types of "necrosis," which we today recognize as apoptosis (Type I necrosis), lysosomal cell death (Type II), and necrosis (Type III). These were identified by morphological criteria, as is described immediately below. The term "programmed cell death" originally referred to the existence of reversible steps clearly leading to the destruction of the cell, operationally defined by experiments. Today programmed cell death more specifically refers to defined genetic pathways in cells but more loosely to apoptotic or other physiological cell deaths. Likewise, the morphological appearance of the apoptotic cell is now understood in more biochemical terms. These distinctions are elaborated in the introduction and elsewhere. They are briefly summarized here to provide relevance to the technical descriptions that follow.

In *apoptosis*, a dying cell loses its adherence for neighboring cells or extracellular matrix, rounds up, and condenses. The chromatin in its nucleus coalesces into one or a few masses along the nuclear membrane, while the cytoplasm, dense by staining with dyes for light or electron microscopy, fragments as the cell forms blebs that are ultimately taken up by phagocytes (Figs. 1 and 2). Mitochondria are normal to shrunken in appearance rather than dilated or swollen, but have depolarized and permitted the escape of cytochrome c and a few other components. The characteristic appearance of the cytoplasm and nucleus results from the activation of preexisting caspases, which can cleave important components of the cytoskeleton and nuclear matrix. DNase II-type endonucleases are activated or gain access to the DNA and cut it between nucleosomes, producing fragments that are identified as a ladder when the DNA is electrophoresed. This pattern of DNA fragmentation is characteristic of apoptosis, but it may reflect failure to degrade fragments further or failure to activate exonucleases rather than specific activation of the endonuclease. Phagocytosis by professional phagocytes or neighboring cells occurs quickly, and the identifiably apoptotic cell has often disappeared within 1 to 2 hours. The phagocytosis is mediated by the appearance on the external face of the cell membrane of phosphatidylserine, actively extruded from the internal face. Apoptosis is a very common process and is seen in the majority of cell deaths, most typically in dying cells that derive from mitotic cell lines and have relatively little cyto-

plasm, such as lymphocytes and thymocytes. Most embryonic deaths are apoptotic, but it is important to realize that both the conversion to apoptotic morphology and the translocation of phosphatidylserine require energy (Schlegel, Callahan, and Williamson, 2000). In situations in which energy (ATP) is compromised, there are severe osmotic considerations (as in the eggs of freshwater organisms), or cell death is so massive that phagocytes cannot rapidly remove all the dying cells (toxicity or genetic problems, perhaps also in regions of poor circulation such as bone), cells may begin to die by an apoptotic mechanism but fail to complete it. In these cases, the cells when recognized may have a very different appearance and the mechanism can be misinterpreted.

*Lysosomal (Type II)* cell death is seen in large, quiescent, or postmitotic cells that have massive cytoplasm, such as glandular tissues (insect glands at metamorphosis, mammary epithelium), muscle, and differentiated neurons. In these cells, the destruction of DNA is not an imperative as it might be for, for example, a potentially mutated or virus- or plasmid-carrying lymphocyte, while cytoplasm is a bulky mass that cannot be simply shed. In these cells, many of the characteristic changes of apoptosis are markedly delayed, and the cell undergoes substantial alteration well before this stage. Most prominent is the appearance of large autophagic vacuoles, lysosomal derivatives that consume the bulk of the cytoplasm. These appear while the cell remains functional and, in the case of muscle, can retain its resting potential (Lockshin and Beaulaton, 1979). Other lytic mechanisms operate as well: Erosion of myofilaments in muscle can occur in these cells external to autophagic vacuoles, most likely in proteasomes (Haas et al., 1995). Autophagy is an intracellular substitute for phagocytosis. Finally, in insects, when approximately 80% of the cytoplasm has been destroyed, the cytoplasm condenses, the chromatin coalesces and marginates, electrophoresis reveals the appearance of a DNA ladder, and the remnants of the cell are phagocytosed as in classic apoptosis (Zakeri et al., 1993). Nevertheless, judging from the few reports extant, the apoptotic phase of death in these circumstances amounts to approximately 10% of the period in which the dying cell is identifiable, and most of the tests for cell death based on characteristics of apoptosis will produce negative results (Jochová, Zakeri, and Lockshin, 1997).

*Necrosis* is an uncontrolled death. If a cell encounters such severe insult or sustains such severe injury that either its ATP-generating mechanisms or the integrity of its permeability barriers is compromised—as might occur, for instance, in infarct, sudden change in pH or osmolarity, or in the presence of several toxins or poisons—then the cell is likely to lyse. The usual scenario is that, in the absence of adequate mitochondrial function and effective ion pumps, the cell switches to glycolysis and accumulates lactic acid. If the cell is not yet severely acidotic, the lactate is trapped within the cell and acts as an osmotic attractant, pulling in water and causing the cell to lyse. This lysis releases into the surrounding tissues many components

including pyrogens and other elements that will, in vertebrates, attract mast cells, leading to inflammation. Necrosis is frequently described as a multicell phenomenon, as opposed to apoptosis as a single-cell phenomenon. However, this description fails to address the situation in which a cell attempts to undergo a dignified and self-effacing apoptosis, but because of circumstances, cannot complete it before succumbing and reverting to necrotic morphology. For instance, in the acutely toxic liver, cells most exposed to the toxin (closest to the central vein) undergo necrosis. The more peripheral cells may undergo apoptosis, or they may begin to undergo apoptosis but finally convert to necrosis. During maturation of bone, resident chondrocytes appear to undergo necrosis, but this may be the outcome of failure of phagocytes to reach them. In plants, there are several types of programmed cell death, including spontaneous deaths, for instance of flowers; deaths activated through oxidative mechanisms or other stresses; deaths induced by infectious agents, apparently as a defense mechanism; and deaths induced by the hypersensitivity of this defense mechanism. In some deaths, for instance, the maturation of tracheal elements, the expansion, and finally collapse of the central vacuole trigger the prompt death of the cell, perhaps by releasing lytic enzymes into the cytoplasm (Fukuda, 1996). Claims for internucleosomal cleavage of double-stranded DNA (Danon et al., 2000) or potentially caspaselike proteases (Lam and del Pozo, 2000) are disputed and the morphologies in general do not resemble either apoptosis or autophagic cell death. Plant cell death is more fully described in the chapter by Mittler and Cheung in this volume.

## Methods Useful to Study Cell Death in Development

Unlike cells in culture, embryos present a challenge for evaluating cell death. Embryonic development represents a dynamic and orchestrated interplay between cell movement, division, differentiation, and death. The cells change constantly in state of differentiation, signals, and position. This complicates analyses in that one has cells in different stages of cell cycle and doing different things next to each other. Although many times in development a group of cells may die in close proximity to each other, it is also possible that individual cells die within a group of otherwise healthy cells. Therefore, many of the methods used in the determination and analysis of cell death for cultured or normally suspended cells (lymphocytes represent the latter category) are not useful or are more difficult to interpret when used in the developing embryo. Although the variety of cells in a tissue and relative paucity of cells of interest in an embryo make the detection of cell death challenging, for the same reasons embryonic development is an excellent system in which to study the signaling systems that regulate an individual cell's decision to die. For instance, the failure of interdigital death and the

consequent webbing of the Hammertoe mouse mutant result not from mutation of a cell death machinery but rather from a failure of the signaling mechanism (Singh Ahuja, James, and Zakeri, 1997; Zakeri and Ahuja, 1994; Zakeri, Quaglino, and Ahuja, 1994b). The advantage in studying cell death in the embryo is that one can identify the dying cells in their spatial and temporal context. For this reason, a number of methods are used to deal with the in situ examination of either complete embryos or embryonic sections on slides. There are several commonly used approaches [these methods have been further described in a review paper (Zakeri and Lockshin, 2002)]. We aim here to present some of the methodology used and to provide some detail, although additional details can be found in the references cited. Images of results using these several techniques are illustrated in Figs. 1 and 2. More than one analytical method should be used to assure correct evaluation of the type or number of dead cells.

## Detection of Cell Death by Morphology

Due to the spatial restriction of deaths of interest in embryos, one of the most useful methods is microscopy. Since for apoptosis at least the morphology is frequently unequivocal, standard techniques for the preparations generally suffice.

## Detection of Cell Death by Light Microscopy

Many stains can be used to detect dead cells by looking for condensed cells in which both the cytoplasm and the condensed nuclei are more darkly stained than the live cells (Fig. 1a). The sensitivity of this method is rather low, but it is useful for sectioned tissues and embryos. There are some variations to the specific steps to use, but generally the method is as follows: embryos are fixed, frozen in OTC® (Miles Laboratory, Elkhard, IN, a cryostat sectioning medium), or paraffin-embedded and sectioned. Sections are brought to room temperature from –70°C, or paraffin-embedded tissue sections are deparaffinized and stained in filtered Harris Hematoxylin (Sigma) for 3 minutes. Slides are then washed in tap water three times for 1 minute each and mounted with Crystal Mount® (Fisher) (Zakeri and Ahuja, 1994). Since embryos contain substantially more water than adult tissues, adequate preservation requires close attention to osmolarity and salt balance of the several solutions.

## Electron Microscopy

Electron microscopy has been one of the most informative methods to identify cell death as well as to examine the state of dying cells and the status of

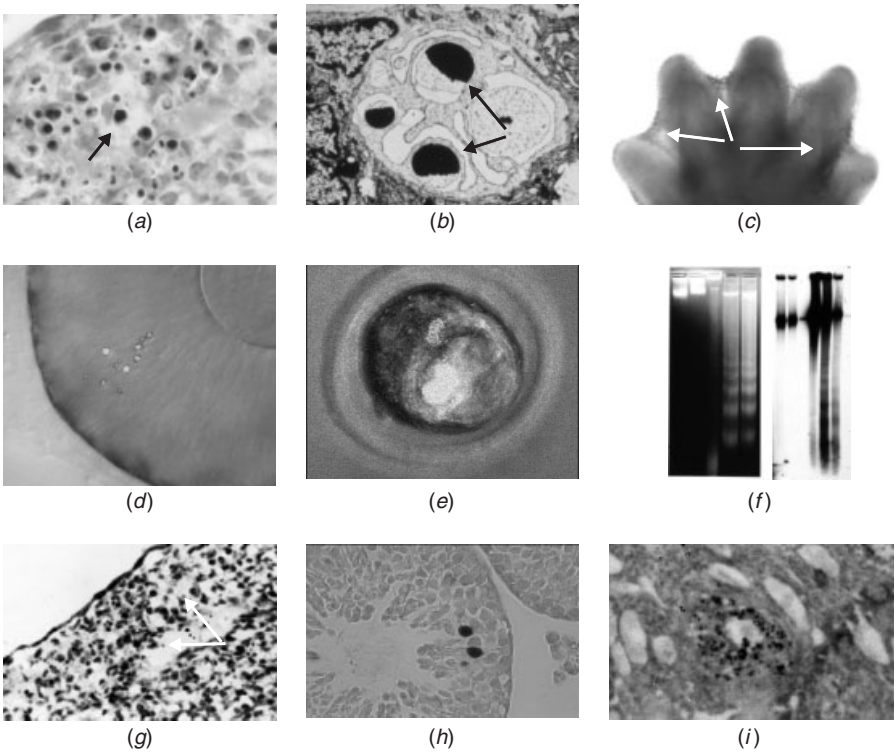



FIGURE 1. See color insert. Examples of several techniques to illustrate cell death or apoptosis. (a) Light microscopy image of haematoxylin and eosin staining of interdigital region of a day 13.5 embryonic mouse hand palette. Darkly stained cells (arrow) are easily distinguished but must be examined at high magnification to confirm that they are not metaphase cells. (b) Apoptotic nuclei (arrows) are readily recognized in the vacuoles of phagocytes (M) in this electron micrograph. (c) Nile blue sulfate is readily taken up into dead cells (actually vacuoles of phagocytes) in the interdigital regions of a day 12.5 embryonic mouse hand palette. Some of the most prominent regions are indicated by arrows. (d) Acridine orange penetrates the stage 17 zebrafish eye. Dead cells in the lens are easily identified by green fluorescence. The phase and fluorescence images were overlaid. (e) Annexin V coupled to a fluor marks the exposed phosphatidylserine on one cell of a mouse blastocyst, as is readily seen by fluorescence using confocal microscopy. The bright green cell is annexin-positive. (f) DNA fragmentation detected by gel electrophoresis using conventional gel (left) and end labeling of the fragmented DNA (right). In both figures, there are two control lanes to the left, and the rightmost lanes are DNA from cells undergoing apoptosis. The ladder seen at lower molecular weights represents fragments of DNA differing in size by 180 bp, indicating that the DNA was cut between nucleosomes. (g) The TUNEL technique, here using the brown DAB-peroxidase reaction revealing dead cells, marked by arrows, in the interdigital regions of a day 13.5 mouse hand palette. (h) DNA fragmentation using TUNEL showing cell death (dark brown cells, TUNEL positive) in the seminiferous tubules of the adult testis. (i) DNA fragmentation using TUNEL showing cell death (dark brown cells) in granulosa cells of atretic follicles of an adult mouse ovary.

different organelles. Typically, mitochondria and endoplasmic reticulum shrink and condense in apoptosis, and the nucleus either rounds or blebs as the chromatin condenses and marginates. In autophagic cell death, the expansion of autophagic vacuoles is prominent, whereas in necrosis mitochondria and endoplasmic reticulum typically swell. The difference between apoptosis and necrosis is described in the introductory chapter, and between apoptosis and autophagic death in the chapter by Bursch et al. Again, there are some variations in the specific method of choice, but basically the steps do not vary much. In brief, samples are fixed in 2.5% glutaraldehyde in 1× PBS at 4°C for several days, and postfixed in 1% osmium tetroxide in 1× PBS at 4°C for 1 hour. They are then dehydrated in graded ethanols and propylene oxide, and finally embedded in Spurr resin. Semithin sections are cut with an ultramicrotome and then can be stained with toluidine blue and observed by light microscopy. These semithin sections provide an excellent overview of cell death in the tissue. Selected areas cut in thin sections are collected on copper grids and stained with uranyl acetate (5% in 70% ethanol) for 15 minutes and then in lead citrate for 10 minutes. The stained sections are examined with an electron microscope (Zakeri et al., 1993). Phagocytosed apoptotic cells are illustrated in Fig. 1b.

## Use of Cellular Changes as Markers of Cell Death

As is described in the introduction to this book, apoptotic cells may be encountered very infrequently even when there is substantial tissue erosion. Thus, techniques that highlight apoptotic cells against a negative background are preferable to those attempting to locate apoptotic cells by appearance alone. Most of the methodology takes advantage of altered organelle function or integrity. These alterations pinpoint molecular and physiological or biochemical alterations within dead or dying cells. They include: in vivo staining of live embryos by vital dyes, and detection of one or more of the following: fragmented DNA; externalized phosphatidylserine; lysosomal activity; and phagocytes, antibody-based or fluorogenic substrate-based techniques to detect activated proteases as opposed to inactive proenzymes or deregulated gene expression. Some laboratories attempt to assess the likelihood of cell death by measuring pro- and antiapoptotic factors such as bcl-2, bax, Fas, and Fas ligand. In the reticuloendothelial system, developmental up- or downregulation of these components may accompany the changing sensitivities of the cells to the level of growth factors as they differentiate. For other cells, however, there is little indication that the fate of the cell is decided by the adjustment of these components as opposed to the signaling mechanism. Thus, these measurements may not always be helpful. Also, for embryos at least, mixed-cell analyses such as flow cytometry and electrophoresis of DNA are of restricted interest unless they can be scaled down

and used for specific tissues after dissociation of the cells from a given tissue and creation of a cell suspension.

## Detection of Cell Death by Vital Staining

Vital stains have long been useful for many developing amniotes such as chicks and mice. The advantage of this method is that it is fast and one can examine cell death in three dimensions with the embryo. However, there are limitations in that one needs a living embryo and the number of detectable dead cells will increase as the embryo dies in culture, leading to false positive results unless the timing and temperature are carefully regulated. For this analysis, fetuses are freed of extraembryonic membranes and stained in a 0.01% solution of Nile blue sulfate (NBS, Sigma) in 1× PBS, for 30 minutes at 37°C with 5% $CO_2$. The dead or dying cells stain dark blue when observed under the microscope, as is illustrated in Fig. 1c. For deep layer observation, it is possible to freeze the embryo in OTC® after vital staining, section, bring to room temperature, cover with a water-based embedding medium such as Crystal Mount® (Fisher), and observe under the microscope. One can detect the deep layer cell death with the Nile blue staining that remains in the cells. This stain indicates the acidic compartments of the cells such as lysosomes, which may be activated in the dying cell, engulfing cell, or both. This method has been used for chick and mouse embryos (Alles and Sulik, 1989; Zakeri, Quaglino, and Ahuja, 1994).

The acridine orange technique when properly used can also be very effective. Acridine orange is classified as a cell permeant but it is only weakly so, and it penetrates nuclei very poorly in healthy cells. Thus, living cells fluoresce a pale green, reflecting the 460 excitation and 650 emission peaks for RNA (it is more orange if the cells contain large amounts of RNA), while at low magnification dead cells fluoresce more brightly, depending on the amount of RNA in the cell, and reflecting the higher penetrance of the dye and its 500 excitation and 526 emission peaks for DNA. With appropriate optics, acridine orange staining can be easily seen, as is illustrated in Fig. 1d. At higher magnification the shape of the nuclei can be readily seen. This method is most effective for embryos at early stages of development, or transparent embryos with few cell layers as penetration of the dye may be a problem. Acridine orange can penetrate dying cells and bind to the DNA much more readily that it can in living cells. Basically, the embryos are submerged in acridine orange (15μg/mL) solution in water for 1 hour at 30°C and rinsed well. The fluorescence can be seen using general-purpose filters in a fluorescence microscope or better with a confocal microscope.

Commercial kits exploit similar possibilities. For instance, several dyes such as calcein AM are cell-permeable and not fluorescent, but inside the cell are cleaved to fluorescent ionic compounds, which are therefore trapped within living cells. Ethidium homodimer I, on the other hand, penetrates

living cells poorly, but penetrates apoptotic or necrotic cells and stains nucleic acids. Live cells therefore fluoresce green and dead ones red-orange. These commercial kits, such as the Live-Dead® kit from Molecular Probes, do not distinguish between apoptotic and necrotic cells. They also may not penetrate whole embryos well and are most effective in cultured cells.

## Detection of Exposed Phosphatidylserine

The use of annexin V labeled with a fluor is usually described as a technique best suited to flow cytometry, but if living cells or embryos can be obtained and examined with a good fluorescence microscope or preferably a confocal microscope, one can detect apoptotic cells as brilliantly fluorescent cells in a dark background. We have had excellent results with both mammalian (mouse) embryos and zebrafish embryos. The penetration of the protein into a whole embryo can be a problem, and false negatives are possible even where some positive cells are detected. Alternatively, annexin V can theoretically penetrate and stain ruptured cells to produce false positives, although in practice this is either uncommon enough or so obvious (i.e., the entire embryo is stained) that it is not a major problem. As is the case for vital staining, the embryo needs to be alive when using this method. The embryo is placed in a diluted mixture of annexin V as described by the vendor for 10 minutes. The staining is visualized by confocal microscopy, as is the case for Fig. 1e.

## Measurement of Fragmented DNA by Gel Electrophoresis

As stated above, one of the hallmarks of apoptosis is the regulated fragmentation of DNA. To detect this fragmentation, different methods can be used. The method most frequently used for the detection of fragmented DNA is gel electrophoresis to separate the fragmented DNA. This method works extremely well for cultured or freely suspended cells such as lymphocytes taken from fresh blood or ascites fluid. However, this is not the method of choice for the study of cell death in most developing systems. It can be used in situations when an entire tissue or organ more or less synchronously undergoes cell death such as is seen in metamorphosing insect or frog tissues. In situations such as interdigital death in the limbs of mammals or chicks, if one can carefully isolate the area undergoing massive cell death, this method may be used. However, the efficiency of detection of the signal is not very good—one needs minimally 30% of the cells at the appropriate stage of apoptosis (Zakeri et al., 1993). This method can work if embryonic tissues are induced to die, as may be the case when embryos are exposed to cyclophosphamide as a model teratogen, and 85 to 90% of the cells die (Zhu et al., 2002). For this method, the tissue is dissected and imme-

diately digested with 0.6 µg/µl proteinase-K (Jersey Lab and Glove Supply) in 50 *mM* Tris buffer with 100 *mM* EDTA and 0.5% SDS) overnight at 37°C. The mixture is treated with 33 µg/mL RNase A (Jersey Lab and Glove Supply), extracted with saturated phenol (Boehringer Mannheim), and loaded onto 2% agarose gels together with ethidium bromide. To increase the sensitivity of the detection, one can enrich for the small molecular weight DNA by lysing the tissue in lysis buffer (0.2% Triton X-100, 10 *mM* Tris-HCl, and 10 *mM* EDTA, pH 7.5). The cell lysate is then held on ice for 15 minutes and centrifuged at 4°C at 12,000 × g for 20 minutes. The supernatant contains the low molecular weight DNA and the pellet contains the high molecular weight DNA. The supernatant is incubated for 1 hour with RNase and then extracted twice with phenol : chloroform : isoamyl alcohol (24 : 24 : 1) and once with chloroform : isoamyl alcohol (24 : 1). The DNA is precipitated with 300 *mM* NaCl and 2.5 volumes ethanol at –20°C overnight. The fragmented DNA is visualized by gel electrophoresis. After electrophoresis the gels are viewed and photographed under UV light (Fig. 1f, left; Karasavvas et al., 1996). In some cases, either because the number of dying cells is low or because DNA fragmentation occurs very late (Zakeri et al., 1993), one cannot detect the DNA fragmentation even with this extraction and enrichment procedure. To increase this enrichment, one can use tritiated thymidine to label the 3′ end of the fragments and then run the gel and expose it to film or an electronic sensor. In this way, the sensitivity is increased at least 10-fold, as is illustrated in Fig. 1f, right (Zakeri et al., 1993; see also Karasavvas et al., 1996).

## The TUNEL Technique

TUNEL is an acronym for terminal deoxyuridine nucleotide end labeling, and as one might expect, in principle any free 3′ end of DNA could be labeled. The basic idea is to identify cells with fragmented DNA. Indeed, it is possible to see TUNEL labeling in necrotic cells (Frankfurt et al., 1996) and in S-phase mitotic cells, particularly those of high chromosome number (Halaby, Zakeri, and Lockshin, 1994). The distinction appears to be that most DNA fragments are extracted from necrotic cells and the number of Okazaki fragments in S-phase cells is much less than the number of free ends in an apoptotic cell. Thus, TUNEL labeling is relative, although usually sufficiently differential to be reliable as an assay. Thorough positive and negative controls are a must. A positive control would be to subject the slide to DNase before the TUNEL technique, while the negative control would be the omission of terminal transferase. This technique, which relies on the action of exogenous terminal transferase to endogenous fragmented DNA, can be used for frozen sections and paraffin sections, fixed by a variety of techniques including ethanol/acetic acid. Several kits are available. We have used a nonisotopic DNA end labeling in situ technique, employing digoxi-

genin-11-dUTP and terminal transferase [ApopTag™ Peroxidase Kit, Oncor, Gaithersburg, MD (Zakeri, Quaglino, and Ahuja, 1994; Zakeri and Ahuja, 1994) or TUNEL POD (Roche Diagnostics, Mannheim, Germany]. Briefly, sections are postfixed in ethanol: acetic acid (2:1) for 5 minutes at –20°C and washed in 1× PBS twice for 5 minutes each. Endogenous peroxidase is quenched with 0.1% hydrogen peroxide in 1× PBS for 20 minutes, and the sections are then rinsed in 1× PBS twice for 5 minutes. Sections are equilibrated in equilibration buffer for 20 minutes before the addition of reaction buffer containing TdT (terminal deoxynucleotidyl transferase) enzyme and digoxigenin-11-dUTP or FITC-DUTP. The reaction is stopped and the label is detected using the appropriate detection system. Slides are counterstained and mounted with Permount® for visualization. The fragmented DNA within the dying cell is detected as a dark red to brown staining in the cell, as is illustrated in the interdigital cells of a mouse embryo (Fig. 1g, h, and i).

The staining sometimes appears on the surface of the cell rather than within the nuclei. This method detects only cells in late apoptosis, when the DNA fragments. Cells at the early stage of cell death or cells in which the nuclei are not in the plane of section may not be identified.

## Comet Assays

In situations where few cells are available but they can be suspended or otherwise freed from the matrix, the comet assay is worth exploring. Like the TUNEL assay, it depends on the retention by apoptotic cells of fragmented DNA, but in this instance individual cells are embedded in agar and subjected to electric fields. Fragmented DNA can be forced from the cell and, when stained with acridine orange or Hoechst dye, it appears as a comet tail to the cell's comet head. Intact DNA moves much more slowly, if at all, and the much smaller fragments from necrotic cells diffuse rapidly from the cell (Benitez-Bribiesca, 1998). This technique is useful where small but adequate numbers of cells can be isolated for study.

## Detection of Lysosomes and Autophagic Vacuoles

For classically apoptotic cells, cytochemical examination of lysosomal enzymes highlights phagocytes, which can be identified as containing apoptotic cell fragments. In other situations, autophagic cells can also be highlighted by the density and size of their autophagic vacuoles. Acid phosphatase is a simple and reliable screening enzyme that survives cryostat sectioning and even, with care, paraffin embedding. It is readily assayed by the use of naphthol-based substrates that couple with fast garnet to form insoluble tetrahydrofuran compounds. Other lysosomal enzymes can be assayed using comparable substrates. However, not all phagocytes or phagocytic or autophagic vacuoles test positive for acid phosphatase, and other lysosomal

enzymes, such as β-glucuronidase, could be tested. Positive acid phosphatase results are illustrated in Fig. 2a.

This assay is useful in many situations, as the role of autophagy is frequently seriously underestimated. To give three examples: Involution of mammary epithelium is often used as an example of apoptosis, but autophagy is well documented as a major process in these cells (Helminen, Ericsson, and Orrenius, 1968; Helminen and Ericsson, 1970, 1971); likewise, involuting prostate epithelium is a classic example of apoptosis, but a major upregulated gene in both involuting prostate and involuting mammary gland is the lysosomal enzyme cathepsin B (Guenette and Tenniswood, 1994); and, lastly, although caspaselike enzymes are highly conserved, have been identified in *Drosophila*, and are considered to be major players in apoptosis in *Drosophila* embryos (Dorstyn, Kinoshita, and Kumar, 1998; Dorstyn et al., 1999a, b), the death at metamorphosis of the labial glands of the hawkmoth *Manduca sexta* and apparently of the salivary glands of *Drosophila* is mediated by proteases other than caspases (*Facey and Lockshin, in preparation*). Autophagy is prominent in other situations as well (Klionsky and Emr, 2000; Stoka et al., 2001).

To measure lysosomal activity, one can analyze acid phosphatase by the use of a kit (Sigma, 180-A) among other lysosomal enzymes (Zakeri, Quaglino, and Ahuja, 1994). Frozen sections are fixed in formaldehyde, postfixed with citrate (pH 3.6)-acetone-37% formaldehyde (13:33:4) for 30 seconds, washed in $dH_2O$, and treated with naphthol AS-BI phosphate and fast garnet stain for 1 hour at 37°C. Slides are washed, air-dried, counterstained with methylene blue, and mounted with Crystal Mount (Fisher). The acid phosphatase activity is detected as a distinct red focal precipitate. Paraffin embedding is also satisfactory, although the activity of acid phosphatase, measured in deparaffinized sections brought to $H_2O$, is substantially lower.

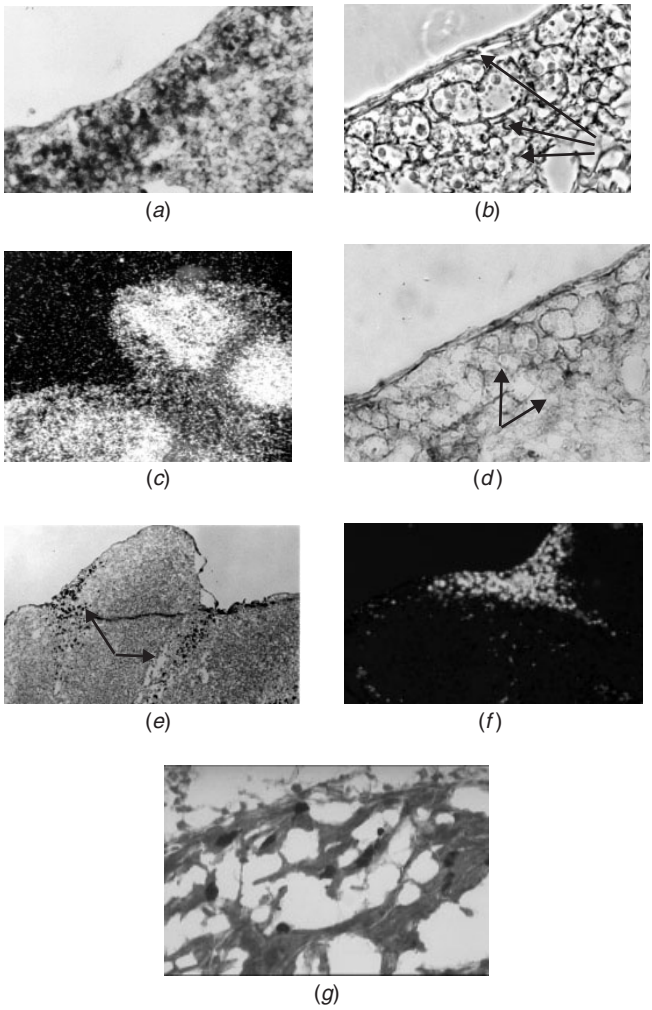
## Detection of Phagocytes

Apoptotic cells persist very transiently, primarily because they are readily identified as prey for professional or amateur phagocytes. Both types of phagocytes may be readily identified by a cell surface marker recognized by a commercially available antibody. Although this technique detects phagocytes, not apoptotic cells, the phagocytes are large and their vacuoles can be readily seen at higher magnifications. The cell boundaries of a macrophage are labeled in Fig. 2b. Counterstaining or dual labeling frequently reveals, within the vacuole, dense blebs of cytoplasm or dense nuclear fragments containing highly condensed and marginated chromatin. Both types of inclusion reveal the fate of an apoptotic cell, although it is usually not possible to make any assumption about the number of cells consumed by a phagocyte or the number of phagocytes containing parts of a single cell. However, one

can use this marker to assess the level of cell death and/or the fate of dead cells in developing systems.

It is also possible to see situations in which the dead or dying cell is not engulfed by the phagocytic cells. This occurs in some abnormal situations such as that caused by an insult in which the level of cell death is very high, that is, cyclophosphamide-treated embryos (Zhu et al., 2002).

Phagocytic cells are detected by standard immunohistochemical procedures. Frozen sections are brought to room temperature and rehydrated. Endogenous peroxidase is inactivated by treating sections with hydrogen peroxide [1 $H_2O_2$ (30%, Sigma) : 2 $H_2O$]. Nonspecific binding sites are blocked with 5% dry milk, and primary antibody F4/80 (Serotec; Hume, Perry, and



(*a*) (*b*) (*c*) (*d*) (*e*) (*f*) (*g*)

Gordon, 1984) diluted at 1/10 in 1× PBST-gelatin is applied for overnight incubation at 4°C. A secondary antibody, a peroxidase labeled $F(ab')_2$ fragment of goat antirat IgG (H + L) at a dilution of 1/50 (Jackson Immunological) in 1× PBST-gelatin, is applied and slides are incubated for 2 hours at room temperature (RT). After washes immunoreactivity is visualized by DAB staining. Slides are counterstained with hematoxylin (Sigma) and mounted with Crystal Mount® (Zakeri, Quaglino, and Ahuja, 1994).

## Detection of Deregulation of Certain Gene Products During Cell Death

A characteristic of truly programmed cell death, defined in 1966 for metamorphosing tadpoles (Tata, 1966), in 1969 for insects (Lockshin, 1969), and later for glucocorticoid-treated thymocytes (Makman, Dvorkin, and White, 1966; Munck, 1971) and trophin-deprived neurons (Oppenheim et al., 1990) is a requirement for protein synthesis. The required proteins are not yet known and therefore not identified as members of any specific pathway of apoptosis. This situation is common in embryonic or developmental situations. Species differences, as between web-footed and other birds, and abnormalities such as cleft palate or hammertoe also indicate that cell death is under genetic regulation (Singh Ahuja, James, and Zakeri, 1997), and, of course, studies on nematodes documented several genes both responsible for cell death and for preventing it. Genetic regulation can theoretically be manifested in the differential expression of new gene product, differential expression of already present gene products, or differential activation or

FIGURE 2. See color insert. Further examples of techniques to illustrate cell death or apoptosis, continued. (a) Interdigital region of the mouse hand palette has been incubated in the presence of a naphthol AS-BT phosphate substrate. The magenta-to-purple color marks high acid phosphatase activity. (b) F4/80 antibody identifies the cell membrane of a macrophage, as indicated by the arrows, in a mouse embryo hand palette. (c) In situ hybridization for bcl-2 message in a developing mouse digit reveals substantial labeling over the cartilaginous areas, but there is no difference in the level of labeling in the interdigital region in which cell death is present compared to the adjacent regions, suggesting that there is no change in the level of Bcl-2 in this region. (d) Immunohistochemistry reveals upregulation of transglutaminase protein (arrows) in dying cells in a mouse hand palette. Transglutaminase is often a good marker for cell death (Piacentini et al., 1991). (e) Although most proteins do not change much in amount, immunohistochemistry can reveal activation of enzymes such as Cdk5, illustrated here (arrows), from a day 13.5 mouse embryo hand palette. (f) Double staining of DNA fragmentation (TUNEL, green) and Cdk5 protein (red) merged (yellow) in a confocal image of a section of the interdigital region of a day 13.5 mouse hand palette. (g) Using immunohistochemistry to show the activation of caspase 3 using an antibody specific to the active form. The dark staining reveals the activation of caspase 3 in the gestation day 13.5 mouse liver.

silencing of gene products. To examine these possibilities during cell death in developing systems, efforts have focused on the regulation of known cell-death-related genes as well as unknown genes. It is sometimes possible to observe a changed level of transcription by in situ hybridization (see Ahuja, Tenniswood, and Zakeri, 1996 for detailed methodology).

In most (nondevelopmental) instances, the commitment to die is not accompanied by a transcriptional or translational change, but rather by the activation of a proenzyme or otherwise dormant enzymatic activity. In fact, in many situations, particularly those not involving physiological cell death mechanisms in embryos, the program is preinstalled in the cell: Protein synthesis is not required and apoptosis may even be induced by treatment with the protein synthesis inhibitor cycloheximide. Given this situation, measurements of transcriptional or translational changes are subject to skepticism. Most changes, if they involve changes in the machinery as opposed to changes in environment (pH, ionic composition) that alter the activity of enzymes, must result from activation or inactivation of enzymes. Some of these changes are known and should be investigated.

## Detection of mRNA by in situ Hybridization

The basic method for in situ hybridization can be found in Ahuja, Tenniswood, and Zakeri (1996) and Singh Ahuja, Zhu, and Zakeri, 1997). The in situ hybridization is described in the same reference. Paraformaldehyde postfixed sections are incubated with proteinase K in PK buffer (1 *M* Tris, 0.5 *M* EDTA). After dehydration in graded ethanol, sections are hybridized with hybridization buffer and the probe in 10 *mM* DTT. Sections are then washed in 5× SSC with 10 *mM* DTT, in 50% formamide in 2× SSC, in 1× washing solution [23.4 g NaCl, 10 mL 1 *M* Tris (pH 7.5) with 5 *mM* EDTA], in 20 mg/mL RNase A in 1× washing solution, in 1× washing solution, in 2× SSC, and 0.1× SSC. Finally, the dehydrated sections are dipped in photographic NTB-2 emulsion (Kodak) and exposed for 1 to 2 weeks, after which they are developed, counterstained with 0.2% toluidine blue, dehydrated, and mounted with Permount. As illustrated in Fig. 2c, one can identify cell populations that express a specific gene, in this case Bcl-2.

## Detection of Changes in Levels of Proteins or Activation of Enzymes

Many changes that occur in dying cells involve posttranslational changes such as aggregation or proteolytic cleavage. These changes have been detected electrophoretically as a decrease in molecular weight, for instance as in the conversion of a procaspase to an active caspase or cleavage of a presumptive caspase substrate. Such procedures are typically not applicable to normally developing embryos. However, immunohistochemistry has often proved useful in revealing changes in the amounts of active forms of several

apoptosis-related proteins such transglutaminase (Fig. 2d) or Cdk5 (Fig. 2e). Another example is an antibody, which recently became available through commercial sources (Cell Signaling Technology, Beverly, MA; New England Biolabs, Beverly, MA), that is purported specifically to recognize activated caspase 3 (Fig. 2g). Another antibody is purported to identify free nucleosomes and can be used in an ELISA procedure, again on the assumption that there are sufficient numbers of cell deaths to detect. Immunohistochemical techniques have also demonstrated an association of proteins previously associated with apoptosis, such as cyclin-dependent kinase 5 (Qi et al., 1995; Zhu et al., 2002). The association, detected primarily by immunohistochemistry, is a posttranslational change (Singh Ahuja, Zhu, and Zakeri, 1997; Zhu et al., 2002).

Another technique of some interest is the detection of proteolytic activity in living cells by the use of cell-permeant fluorogenic substrates. The fluorescent cleavage product is ionic and remains trapped at least through the early phases of apoptosis (Komoriya et al., 2000). The one most commonly used is a substrate for caspase 3. Caspase 3 is the major effector caspase, meaning that it is activated by an initiator caspase (caspase 8 or 9, themselves resident in cells as proenzymes) and digests major intracellular components of the cytoskeleton and nuclear matrix. Although they have not yet been used extensively for embryos, these cell-penetrant fluorogenic substrates are promising. In preliminary experiments we have seen that the fluorogenic substrate can identify in situ cells dying in zebrafish embryos.

These techniques can be used to assess apoptosis in abnormal situations, whether locally as a result of mutation (e.g., hammertoe mutation in mice—Zakeri, Quaglino, and Ahuja, 1994a) or massive as a result of treatment with a strong teratogen such as camptothecin (Mirkes, 1985) or an inducer of apoptosis such as cycloheximide (Hensey and Gautier, 1997). One may also learn in a generic manner from knockouts, as for instance in the case of a caspase-9 knockout in mice although, as mentioned above, the phenotype may be more complex than anticipated (Oppenheim et al., 2001).

## Immunohistochemistry and DNA Fragmentation Double Labeling

Fluorescence detection of DNA fragmentation and Cdk5 expression can be used as in Singh Ahuja, Zhu, and Zakeri (1997) and Zhang et al. (1997). Sections are processed according to the instructions for the Apoptag® kit (Intergen, Purchase, NY) with FITC fluorescein labeling, followed by two washes with PBST. We have used primary anti-Cdk5 antibody at a final concentration of 1 µg/mL with secondary biotinylated antibody and finally have incubated the slides with cy3-conjugated IgG mouse antibiotin (Jackson Immuno Research Laboratory, West Grove, PA) for 30 minutes. The slides are mounted with 90% glycerol. DNA fragmentation is seen with FITC as green and the Cdk5 as detected by cy3 is red.

## Conclusions

Cell death is a prominent part of development and historically has been the source of ideas such as that of programmed cell death, the existence of cell death genes, a cell death pathway, and the role of caspases. The study of cell death in embryos could produce much more, including an understanding of the subtle mechanisms controlling patterning of cell death and the important cell-to-cell and tissue-to-tissue interactions as well as the molecules defining the ability to undergo apoptosis, to name two obvious examples. Limitations include the small size and regional specificity of embryos. The most functional techniques for studying embryonic cell death are primarily microscopic; other techniques are possible following microsurgery. Genetic analysis using knockout or overexpression systems is sometimes limited by the ability of embryos to make use of redundant pathways, but has nevertheless provided much information about the function of cell death. Transiently controlled alteration of expression is likely to produce more meaningful results. Future researchers will hopefully exploit the unique possibilities in embryos to lead us to a more profound understanding of both embryonic development and the mechanisms of cell death.

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CHAPTER

3

# CELL DEATH IN *DICTYOSTELIUM*: ASSESSING A GENETIC APPROACH

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## CASPASE-INDEPENDENT MECHANISMS OF DEATH, THE CASE FOR NONANIMAL MODELS, AND THE CHOICE OF *DICTYOSTELIUM*

We animals are the exception. Not that animals are the only beings to exhibit cell death; far from it, cell death pervades life, and can probably be found, provided it is looked for, in every multicellular organism of every kingdom of life. However, animals alone (Uren et al., 2000) exhibit a caspase-dependent mechanism of death (Ellis and Horvitz, 1986), which translates morphologically into apoptosis (Kerr et al., 1972).

Animal cells can also harbor caspase-independent mechanisms of cell death, leading to nonapoptotic, necrotic (Fiers et al., 1999; Searle et al., 1982), or vacuolar (Clarke, 1990; Schwartz et al., 1993; Zakeri et al., 1995) cell death. Such underlying mechanisms can surface in particular if and when caspase activation is impaired or prevented, be it for experimental (Vercammen et al., 1998), genetic (Chautan et al., 1999), or pathological reasons such as infections. Indeed, some pathogens can encode molecules interfering with

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caspase activation (Bump et al., 1995; Clem and Miller, 1994; Hawkins et al., 1996). Thus, caspase-independent cell death may well occur in pathological situations. This calls for convenient methods, faster than electron microscopy, equivalent to the TUNEL technique for caspase-dependent cell death, to detect this sort of cell death. However, no such method is currently available, which stems from a general lack of knowledge as to the mechanisms of caspase-independent cell death, which in turn calls for further efforts to elucidate these mechanisms.

In which experimental model should one study caspase-independent mechanisms? Since genuine caspase-encoding genes have been found only in animals (Uren et al., 2000), it follows that cell death outside the animal kingdom must be based on caspase-independent mechanisms. It is conceivable that caspase-independent mechanisms of cell death might be conserved in evolution, and that some of these mechanisms could be shared between nonanimals and animals. Establishing bases of cell death in nonanimals might thus provide helpful hints as to caspase-independent mechanisms of cell death in us. Which nonanimal model might then be of convenience, in particular of genetic convenience? Among several appealing possibilities (such as the Alga *Volvox*; Tam and Kirk, 1991), the protist *Dictyostelium discoideum* strikes us as especially promising.

*Dictyostelium* seems to have emerged in evolution after divergence of the kingdom Plantae and before individualization of the kingdoms Animalia and Fungi (Baldauf et al., 2000). Demonstration of a common cell death mechanism between this organism and some of the higher eukaryotes would be a strong argument for a degree of generality of this mechanism. Also, the study of cell death occurring during *Dictyostelium* development should be facilitated by the relatively simple pattern of this development: Upon starvation, isolated *Dictyostelium* cells aggregate, differentiate, and morphogenize into 1 to 2 mm high multicellular fruiting bodies. Each of these contains a mass of spores supported by a stalk. Cells in the stalk resulting from starvation-induced *Dictyostelium* development have been considered dead on the basis of nonregrowth when these cells were incubated in a rich medium (Whittingham and Raper, 1960). This developmental cell death can be mimicked in vitro using *Dictyostelium discoideum* mutant cells HMX44A that, upon starvation and the addition of DIF, differentiate as a monolayer from vegetative to "stalk" vacuolated dead cells (Kay, 1987; Cornillon et al., 1994), thus facilitating the isolation of dying *Dictyostelium* cells for study. The genome of *Dictyostelium* is small ($\sim 3.4 \times 10^7$ base pairs; about 100-fold smaller than that of higher eukaryotes), its sequencing is currently approaching completion, and it is haploid. Genome haploidy makes it relatively easy to generate and select mutants of function-associated genes and to identify the latter (Kuspa and Loomis, 1992; Loomis, 1987; Kuspa et al., 1995). Because of the temporal separation between vegetative growth and development, developmental mutants (such as those related to cell death) can be propa-

gated under vegetative conditions, thus behaving like conditional mutants (Loomis, 1987). Finally, and more trivially, *Dictyostelium* cells can be grown in large quantities on inexpensive media, are robust, and have been a popular model system for the biochemical and physiological analysis of signal transduction for many years. Wild *Dictyostelium*, which are found in decaying leaf litter, feed on bacteria. While the initial laboratory strains required bacteria to feed on, axenic strains have been derived, which greatly facilitates in vitro work.

Altogether, one proposes to analyze cell death in an organism phylogenetically relatively distant from the usual models used to study cell death, simpler, and endowed with at least equivalent genetic advantages. This might allow one to define molecular bases of cell death, which might be experimentally accessible in this organism more than in others.

We wish here, after a brief survey of the main features of Dictyostelium cell death, to describe in some detail a genetic approach aimed at defining its molecular bases, to list the main results obtained, and to critically assess this approach in comparison with similar attempts in other organisms.

## Main Features of *Dictyostelium* Cell Death

Developmental cell death in *Dictyostelium* is a caspase-independent and morphologically vacuolar cell death. Let us consider in more detail occurrence, caspase independence, and phenomenology, respectively.

Although *Dictyostelium* cells have been observed to die also at the unicellular stage when constrained (Tatischeff et al., 2001), to focus on naturally occurring cell death, we investigated only developmental cell death, that is, cell death observed in the stalk upon starvation-induced development, or a likely in vitro equivalent thereof. Stalk cells were known to be vacuolated (de Chastellier and Ryter, 1977; George et al., 1972; Maeda and Takeuchi, 1969; Quiviger et al., 1980; Raper and Fennell, 1952; Schaap et al., 1981) and were considered nonviable according to the criterium of nonregrowth in a culture medium (Whittingham and Raper, 1960). Differentiation to stalk cells seems to result from the sequential action of at least two factors, that is, cyclic AMP (cAMP) promoting in particular cell aggregation, and a factor called DIF promoting in particular the differentiation of starved cAMP-subjected cells to stalk cells (Morris et al., 1987; Sobolewski et al., 1983; Town and Stanford, 1979; Town et al., 1976).

Since it is not convenient to study cell death in stalks, we used a model system that in many respect behaves as an in vitro equivalent of stalk cell death in vivo. HMX44, an axenic mutant derived from V12M2, not producing DIF but responding to exogenously added DIF, was obtained from J. G. Williams (University of Dundee, Dundee, Scotland). The subclone HMX44A was derived and used in this laboratory. As stated above, *Dictyostelium*

HMX44A cells upon starvation and the addition of the differentiation factor DIF differentiate as a monolayer from vegetative to "stalk" vacuolated dead cells (Cornillon et al., 1994; Kay, 1987). Both starvation and the presence of DIF were required for cell death. Since DIF did not induce the death of vegetative cells, it follows that it can induce *Dictyostelium* death only when added to cells that have already reached a certain level of differentiation. More generally, in agreement with previous work (Kay, 1987), under our experimental conditions cell death occurred only when a given program of successive extracellular signals, that is, starvation and DIF, was applied to the cells (not shown). In any case, the use of these HMX44A cells enables one to distinguish between the effect of starvation alone, not leading to cell death for at least 3 to 4 days, and the effect of starvation plus DIF leading within 10 to 12 hours to irreversible block of the ability to regrow (Cornillon et al., 1994). Although in vitro DIF is strictly required for the induction of HMX44A cell death, in vivo DIF is required only with regard to one of the major prestalk cell types, the pstO cells (Thompson and Kay, 2000).

We investigated whether cell death depended on caspases (Olie et al., 1998). Even high concentrations of the caspase inhibitors zVADfmk, BOC-Asp-fmk, DEVDfmk, or YVADcmk, added together with DIF, did not lead to the inhibition of cell death as assessed both morphologically and by cell counts after regrowth. Absence of inhibition of cell death by caspase inhibitors suggested that *Dictyostelium* cell death is not dependent on caspase activation (Olie et al., 1998). More recently, no genuine caspase gene could be found in the *Dictyostelium* genome (Uren et al., 2000), strongly supporting the conclusion that *Dictyostelium* cell death is caspase-independent. Interestingly, and controlling in part for the caspase inhibitor experiments, cyclosporin A and the general serine- and cysteine-protease inhibitor TPCK inhibited *Dictyostelium* cell death (Olie et al., 1998). From another point of view, the addition of high concentrations of caspase inhibitors on developmentally competent *Dictyostelium* AX2 cells resulted upon induction of development in a sharp decrease in the proportion of complete sorocarps, and to a correlative increase in the percentage of aggregates/culminants and (near)stalk-less elements. This effect was dependent on the concentration of DEVDfmk or YVADcmk, whereas the addition of zVADfmk or BAF had no detectable effect at all concentrations tested (Olie et al., 1998). It could well be that caspase inhibitors affected nonspecifically other proteases (Schotte et al., 1999) that are required at a predeath stage of *Dictyostelium* development.

Phenomenologically, in temporal succession after the triggering of differentiation, PCD included first an irreversible step leading to the inability of about 50% of the cells to regrow in 8 to 12 hours. If nonregrowth means that an irreversible event has occurred in the differentiating cell, then clearly this irreversible event occurred significantly before any morphological alteration detectable by the techniques used here. Then, at 12 to 14 hours,

increased vacuolization was best evidenced by confocal microscopy, and prominent cytoplasmic condensation and focal chromatin condensation could be observed by electron microscopy. Membrane permeabilization occurred only very late (at 40–60 hours) as judged by propidium iodide staining. No early DNA fragmentation could be detected by standard or pulse field gel electrophoresis. DNA from *Dictyostelium* cells subjected to DIF for 12 or 24 hours showed no sign of massive degradation, that is, neither small nor large DNA fragments, nor marked smears (Cornillon et al., 1994). It was recently confirmed that *Dictyostelium* cell death showed no oligonucleosomal DNA degradation as assessed by agarose gel electrophoresis (Arnoult et al., 2001). The more marked smear obtained with 66-hour cells also occurred in control groups without DIF (Cornillon et al., 1994). Interestingly, *Dictyostelium* cell death was accompanied with a marked decrease of mitochondrial membrane potential, and cells could be labeled with Annexin V, suggesting phosphatidylserine externalization, however relatively late in the process (Arnoult et al., 2001).

By electron microscopy, vegetatively growing *Dictyostelium* cells showed in particular a highly developed cytoplasmic system of small vacuoles and a nucleus with a large nucleolus associated with the nuclear membrane and homogeneously dispersed chromatin without condensation. After 8 to 12 hours of starvation without DIF, spots of condensed chromatin were visible within the nucleus, with significant cytoplasmic condensation (Cornillon et al., 1994). Thus, perhaps surprisingly, cells starved with or without DIF showed similar aspects of chromatin and cytoplasmic condensation, different from those of vegetative cells. Since HMX44A cells do not immediately die when starved without DIF, the results above indicated that the observed levels of cytoplasmic and chromatin condensation are not enough to ensure PCD in *Dictyostelium* (Cornillon et al., 1994). Similar nuclear alterations were one of the arguments that AIF, released from mitochondria and translocated to the nucleus, might be involved in *Dictyostelium* cell death (Arnoult et al., 2001).

After 14 hours of incubation in the presence of DIF, the most obvious difference with cells starved without DIF was the presence of large cytoplasmic vacuoles, either appearing empty or containing residual material. Thus, a major feature of cell death in *Dictyostelium* is vacuolization. Vacuolization could be triggered independently of cell death but by the same sequence of extracellular events, and might then be related to differentiation toward an aspect reminiscent of that of plant cells or fungi. Alternatively, vacuolization may be directly related to cell death, either as a cause or consequence; the latter seems more likely, considering that the inability to regrow occurs on average a few hours earlier than detectable vacuolization. Electron microscopy studies indicated that in developing *Dictyostelium*, vacuoles may be related to autophagy (de Chastellier and Ryter, 1977; George et al., 1972; Maeda and Takeuchi, 1969; Quiviger et al., 1980; Schaap

et al., 1981). However, the presence of large vacuoles cannot be just a reflection of an autophagic process secondary to the incubation in starvation medium (as observed in some mutant strains of yeast cells; Takeshige et al., 1992), since HMX44 cells starved without DIF do not show large vacuoles.

This phenomenological analysis has revealed several features of *Dictyostelium* cell death, some of which are encountered in many other cases of cell death, whereas some other features are more restricted. However—and perhaps not surprisingly—this has not provided much information as to mechanisms at play. After all, the features of cell death are a function of mechanical constraints and preexisting enzyme stores perhaps more than of nature of molecular triggers. We view these results more as an investment for the molecular future, when known phenomenological landmarks will make it easier to functionally map candidate molecules. But how to identify these?

## A Genetic Approach to the Molecular Mechanism of *Dictyostelium* Cell Death: Method

As stated above, a mutagenesis approach in *Dictyostelium* benefits from *Dictyostelium*'s haploidy; moreover, developmental mutants sparing the vegetative stage are conditional. On the basis of this background, insertional mutagenesis offers additional advantages. Insertional mutagenesis involves introducing in *Dictyostelium* a plasmid, which will integrate in the genome and thus may in very rare cells disrupt a gene involved in the function of interest. Insertion is believed to be random: This approach is not biased by preconceived ideas as to possible mechanisms. An obvious advantage over chemical mutagenesis is the possibility, from a selected (in this case, cell-death-resistant) cell, of rescuing the disrupting plasmid together with the flanking genomic sequences, which allows the identification of the disrupted gene.

We obtained transformants through electroporation (Howard et al., 1988) of a plasmid bearing a blasticidin resistance marker (Adachi et al., 1994), using the REMI approach (restriction enzyme-mediated integration) and leading to frequencies of around $4 \times 10^{-5}$ integrating transformations (Kuspa and Loomis, 1992, 1994). In our experiments, transformants were then selected, first for their blasticidin resistance, then for their resistance to cell death induced by starvation and DIF. The resulting cell population was resuspended in a rich axenic medium, in which cell-death-resistant mutants regrew (Levraud et al., 2001). However, even in nontransfected HMX44A cells, upon the induction of cell death and resuspension in a rich medium as many as 10 to 20% of cells will regrow. To enable mutants to emerge from this high background, successive cycles (up to eight) of cell death induction

and regrowth were required (Cornillon et al., 1998). The problems raised by the recycling procedure imposed by a high background are discussed below. If at this stage a cell population is obtained that exhibits significant resistance to cell death, it is cloned. Clones are again checked for resistance to cell death, and the inserted plasmid is rescued, sequenced, and used for homologous recombination on wild-type *Dictyostelium* to check that homologous recombinants show the same resistance phenotype, demonstrating that it was indeed the disruption of this gene that led to the acquisition of this phenotype.

## A Genetic Approach to the Molecular Mechanism of *Dictyostelium* Cell Death: Results

The approach outlined above [and described in detail in Cornillon et al. (1998) and Levraud et al. (2001)] provided cell-death-resistant mutants at a frequency of about $10^{-9}$. Thus, out of about $2 \times 10^{10}$ transfected cells, a total of 16 such mutants were obtained. They were called DDM, for *Dictyostelium* death mutant. In these DDMs, we investigated the relationship between cell death resistance phenotype and intended insertional mutagenesis.

For 12 out of these 16 DDMs, plasmid insertion was irrelevant or impractical, for various reasons. DDM6, 7, and 8 showed inconsistent cell death resistance phenotype. Their study was discontinued prior to any attempt at plasmid rescue. DDM11 showed concatemeric insertion of the plasmid. This not infrequent event (Adachi et al., 1994; Barth et al., 1998) makes plasmid rescue very difficult. The disrupted gene was not identified. For DDM1, 2, 5, 10, and 14, plasmid rescue or PCR-based walking in genomic DNA allowed the identification of sequences flanking the integrated plasmid. These sequences however were coding for sequences that were either irrelevant, or noncoding while apparently not corresponding to regulatory sequences. DDM9, 15, and 16 showed plasmid insertion in a promising open reading frame, but further analysis showed that this insertion was not causal to the cell death resistance phenotype, since homologous recombination using plasmids rescued from these cell-death-resistant mutants did not recapitulate the mutant phenotype.

A further group of three DDMs showed insertions in genes that may be involved in the signaling of cell death. Namely, DDM3 showed a single insertion into a noncoding region upstream of the *RegA* coding region (sequence no. AJ005398), which encodes the cytoplasmic cAMP phosphodiesterase (Shaulsky et al., 1998). The response regulator RegA and the histidine phosphotransfer protein, RdeA, constitute two essential elements in a eukaryotic His-Asp phospho-relay network that regulates *Dictyostelium* development and fruiting body maturation. Comparative northern blot analysis on RNA extracted from HMX44A and DDM3 cells undergoing development showed

the dysregulation of *RegA* in DDM3 cells, which may be due to the insertion and may account for the DDM3 phenotype. In DDM12, insertion of the plasmid was in a MAP kinase phosphatase gene (sequence no. AB018543), which had previously been identified by T. Kon, H. Adachi, and K. Sutoh while working on aggregation minus mutants in *Dictyostelium*. This group is proceeding with the study of this gene. Plasmid insertion in DDM13 was in a gene (sequence no. JC2b141a01) with significant homology to Rab. Altogether, these three insertional mutants showing resistance to cell death had insertions in genes encoding molecules consistent with their possible involvement in cell death signaling. In other words, if the insertions are relevant to the cell death resistance phenotype, it would be for "upstream" signalization rather than at the level of the mechanism of cell death itself. This led us to not attempt homologous recombination or further functional analysis for these genes.

For the last of these 16 DDMs, DDM4, plasmid rescue led to the identification of DlrA, the involvement of which was further validated through homologous recombination. The gene disrupted in DDM4 encodes a protein with a leucine-rich repeat domain. The molecule was named DeliriumA (for *Dictyostelium* leucine-rich repeat molecule A, gene symbol *DlrA*; sequence no. AF272150). The rescued plasmid was transfected into HMX44A cells, leading to disruption of the *DeliriumA* gene via homologous recombination in some transfected cells. These were indeed relatively resistant to cell death, confirming that disruption of the *DeliriumA* gene leads to resistance to cell death. An extensive study further revealed that DeliriumA regulates PKA-C expression by inhibiting a previously unsuspected PKA-C mRNA coding-region control (Adam et al., in preparation). In DlrA- cells, the absence of a PKA-C expression can account at several levels for the absence of developmental cell death.

## A Genetic Approach to the Molecular Mechanism of *Dictyostelium* Cell Death: Critical Discussion

Our first mutagenesis campaign thus provided two main sets of results. First, in the majority of the mutants we obtained, the plasmid insertions were irrelevant to cell death. Second, in those mutants where plasmid insertion was probably or certainly causal of resistance to cell death, signalization rather than mechanism was affected: These few mutants thus provided inadequate answers to our initial question about mechanism (while at least one of these mutants provided unasked for but interesting answers about signalization). Possible explanations for these results are discussed below.

## From High Background to Multiple Cycles of Selection to Unexplained Acquisition of Resistance to Misleading "Mutants"

A problem we identified early when setting up this system was that not all cells died in our assay. This "background," initially thought of as only a practical annoyance, turned out to exert profound influences on the outcome of the screen. As mentioned above, our analysis of cell death in *Dictyostelium* relies on an in vitro assay (Cornillon et al., 1994; Kay, 1987), during which cells can be induced to differentiate into stalk cells and die without undergoing morphogenesis. This convenient assay was also the basis of our mutant screen. What we call the background in this assay corresponds to the fraction of cells that do not die during this process, usually 10 to 15%. This background is measured after a period of regrowth in a rich medium, required to reach a cell density sufficient for counting, by comparing numbers of cells in the experimental group with control cells starved in the same way but in the absence of DIF (Cornillon et al., 1998; Levraud et al., 2001). On a few occasions, this background was also measured using a more rigorous assay based on a loss of clonogenicity, which confirmed that our standard procedure was not biased and did correctly measure the fraction of nondying cells.

A number of approaches to reduce this background, using various drugs or changing culture conditions, were unsuccessful. The origin of this background of cell death resistance in "virgin" cells is a matter of speculation. One may think of cells differentiating along the prespore pathway or of cells that do not differentiate at all; this may be a stochastic process, or may be related to the position of the cell along the cell cycle at the time of starvation. Whatever the cause, it strongly depends on the strain used, as was shown before we started our screen (Berks and Kay, 1988). As far as we know, the cell line we initially chose still appears to be the most appropriate for such experiments.

Following mutagenesis and antibiotic-mediated selection of clones harboring stable integrations, putative cell death mutants may be present in the population, but rare and thus undetectable. Because of the background, bulk differentiation into stalk cells and regrowth of surviving cells result at best in the enrichment of these mutants. The problem was to make cell death-resistant mutant cells emerge from the background. The solution was to repeat several times these cycles of induction of death followed by regrowth in rich medium. At least four cycles are required for a cell death mutant to become quantitatively dominant in the population (if we consider subpopulations of about 3,000 independent clones, and, at best, a 7-fold relative enrichment in mutant cells at each cycle, since 10–15% of wild-type cells also survive the process). Thus, following REMI mutagenesis, polyclonal populations of cells with many different genomic integrations were subjected to

repeated cycles of induction of programmed cell death, followed by regrowth of surviving cells by the addition of culture medium (Cornillon et al., 1998).

The emergence of a mutant in the population may be monitored by measuring the effective background at each cycle (as defined above: ratio of surviving cells in the DIF-treated population, relative to the DIF-less population). A background that would become significantly higher than the 10 to 15% value of wild-type cells is a sign that the mutant cells become numerically dominant in the population. This was the initial procedure we used, leading to the isolation of several mutants (Cornillon et al., 1998). However, cell counts performed at each cycle, if we consider the fact that a large number of independently mutagenized subpopulations have to be treated in parallel, represents a large amount of work, which effectively limits the number of mutants obtained. Cell regrowth was therefore checked only after a given number of cycles had been performed.

Given the expected number of independent clones in a given mutagenized population (c. 3,000 in our usual procedure), and an average fraction of 10 to 15% of wild-type cells that do not respond to the cell death induction protocol, checking for cell survival in each subpopulation only after the eighth differentiation cycle had been performed seemed optimal. The expected result was that subpopulations which did not contain a cell death mutant to start with would still display a wild-type background, whereas subpopulations in which the enrichment of a mutant cell would have taken place would show much higher cell survival.

Thus, further mutagenesis experiments were undertaken, following this "blind" protocol. A surprise came at the end of the process after eight cycles of differentiation and regrowth, as all the cell lines turned out to score as "resistant to cell death." However, control cells, which had not been subjected to mutagenesis but went through the same eight cycles of differentiation and regrowth, turned out to be significantly more resistant to cell death than virgin HMX44A cells as well. This "acquisition of resistance" by nonmutagenized cells was reproducible.

An obvious possibility was that, perhaps because of a spontaneous mutation event, the HMX44A cell line contained a very low frequency of cells with a greater resistance to cell death, and that these rare cells were selected during the process. To test this hypothesis, the cell line was then subcloned, and several clones were subjected to a carefully monitored series of cell death induction/regrowth cycles. The results were clearcut: All these clones, initially quite sensitive to cell death induction (with a normal background), progressively acquired a resistant phenotype, with a background close to 70% instead of the initial 10 to 15%. Thus, resistant cells (called "multistarved" below, by contrast with virgin cells) probably do not preexist in a virgin population, and the phenotypical alteration appears to be induced during the course of multistarvation.

The resistance of the cell line steadily increased with successive cycles, and we wondered whether this was homogeneous (i.e., all cells acquire a progressively more resistant phenotype) or not. One of the cell lines was subcloned while it still displayed only a partial resistant phenotype (background around 45%, after four cycles of differentiation-regrowth). The resistance of ten independent clones was measured: They clearly fell into two groups, some with a wild-type background, some with a multistarved background. Therefore, at the cell level, the multistarved phenotype does not appear to be acquired progressively. Instead, it probably results from an abrupt change of a yet unknown nature, these more resistant cells being then further enriched during the remaining cycles.

This change in resistance to cell death was the clearest phenotypical alteration of multistarved cells. A tendency to aggregate less well than virgin cells was also noted. Stalk cells (based on aspect under phase contrast microscopy, and cellulose staining) end up forming in multistarved cells, but this requires a longer time than for virgin cells. Interestingly, multistarved cells put to vegetative growth for many weeks and then again induced to die still show this much higher resistance to death: The multistarved cell-death-resistant phenotype is thus stable upon a vegetative growth for at least an estimated 160 doublings. Virgin cells also retain a virgin phenotype upon a vegetative culture of comparable duration.

The origins of these phenotypical alterations are, for the moment, a complete mystery. The apparently abrupt change at the cell level would be compatible with a spontaneous mutation, however, of extremely high frequency, which contrasts with the phenotypical stability of *Dictyostelium* cells upon vegetative growth. The alteration may thus rather be of an epigenetic nature, which is not easy to test since the genetic crossing of *Dictyostelium discoideum* strains is very difficult. Another tempting hypothesis is that, perhaps because of starvation, diploidization of some cells occurs during the differentiation cycles, and that diploids are for some reason more resistant to cell death. Such a hypothesis still awaits rigorous testing. Whatever the reason, spontaneous acquisition of a cell-death-resistant phenotype clearly occurs in HMX44A cells subjected to repeated cycles of differentiation and regrowth.

Many of the cell death mutants selected and characterized during the course of our successive screens turned out not to stand the test of reconstitution of the mutagenesis vector insertion in virgin cells: In a majority of cases, the homologous recombinants obtained did not display any resistance to cell death. This is very likely due to acquisition of a multistarved phenotype by the original mutant cells, even though their initial genetic alteration, due to insertion of the vector, did not confer them an increased resistance. Such a possibility at first escaped our attention before blind differentiation-regrowth cycles were performed. Importantly, such a phenotypical alteration would probably not have occurred, had we not been compelled to perform

so many cycles of differentiation and regrowth because of the relatively high background of survival of wild-type cells. "Real" mutants were isolated before the eighth cycle, or were isolated at the eighth cycle, were then sometimes only barely more cell-death-resistant than the multistarved misleading clones, and could unambiguously be distinguished from these only upon homologous recombination. One obvious conclusion is that, when one intends to perform a screen for cell death mutants, the quality of the cell death induction assay is of paramount importance. The higher the background for such a test, the greater the chance of obtaining false positive mutants! Time spent refining the conditions for induction of cell death is thus definitely well spent.

## Poor Adequation of the Few Genuine Insertional Cell-death-resistant Mutants Obtained to the Initial Question about Mechanism

In a few of our mutants in *Dictyostelium*, cell death resistance was indeed causally linked to insertion of the vector. However, the corresponding mutations turned out to block cell death upstream rather than downstream, that is, within signalization pathways rather than in the cell death mechanism proper. Interestingly, this seems to have often been the case when similar genetic approaches were applied to study molecular mechanisms of cell death in other organisms, as discussed below.

In *Drosophila*, although cell death effector molecules akin to those generally found in the animal kingdom were found in silico through searches for homologues (Bangs et al., 2000), some of the molecules at play were first identified through a genetic approach, by exploiting the existence of a number of strains with a range of chromosomal deletions. Embryos of such diverse strains were stained with cell-death-revealing acridin orange, in search of embryos not showing these dead cells. This led to the discovery of several genes required for cell death, namely, Reaper (White et al., 1994), Hid and Grim (Chen et al., 1996; Grether et al., 1995). The products of these genes may be pro-apoptotic through their complexing with the antiapoptotic IAP molecules (Goyal et al., 2000). Their functional (but not structural) homologues in mammals seem to be Smac/Diablo (Du et al., 2000; Verhagen et al., 2000) and Omi/HtrA2 (Martins et al., 2001). Thus, in *Drosophila* a direct genetic approach uncovered molecules apparently restricted to *Drosophila*, and again involved in signaling/regulating rather than effecting cell death.

Not enjoying the genetic advantages of *C. elegans* or *Drosophila*, mammals did not lend themselves easily to a search by classical genetic methods in vivo of molecules involved in cell death. Some such approaches have nevertheless been possible, in vitro, on cell lines, by trying to compensate diploidy with methods generating equivalents of dominant mutations. Thus, the transfection of Hela cells with an antisense cDNA library, followed

by selection for resistance to death, led to the isolation of several gene-encoding molecules required for cell death. The most throughly studied of these is DAP-kinase, a serine/threonine proteine kinase associated with microfilaments (Deiss et al., 1995; Cohen et al., 1997). DAP-kinase is required for cell death induced through Fas or TNFR1 (Cohen et al., 1999), or triggered by TGF-beta (Jang et al., 2002), in both cases interestingly upstream of mitochondrial events. DAP-kinase, a molecule which is clearly important for cell death, identified with the help of an unusual genetic approach, is thus also more involved in signaling than in a direct effector function.

In plants, most of the molecules involved in cell death uncovered so far seem again to deal with signaling, for instance, as resistance genes in the hypersensitivity reaction to pathogens (Bonas and Van den Ackerveken, 1999; Hammond-Kosack and Jones, 1996; Holt et al., 2000). In yeast, a genetic approach led to the discovery of Bl-I, through a search for mutants resistant to death induced by the transfection of Bax (Xu and Reed, 1998). In the fungus *Podospora anserina*, cell death is manifest in incompatible fusions through the death of heterokaryons (Rizet, 1952), genetically controlled by *het* loci (Coustou et al., 1997, 1999; Saupe, 2000). Suppressive or subtractive approaches uncovered other relevant genes (Loubradou et al., 1997; Bourges et al., 1998). Some of these genes seem to be causally involved in cell death, however again more in signaling than in the actual effector mechanism.

The findings in *C. elegans* were somewhat at variance with the above. In this organism, a classical and pioneering genetic approach led to the discovery of key molecules in caspase-dependent apoptotic cell death. Taking advantage of the easy identification of dead cells in this organism by Nomarski microscopy and of favorable genetic circumstances in this hermaphrodite, cell death mutants were obtained through chemical mutagenesis. Such mutants interfered with DNA fragmentation (Sulston, 1976) or dead cell clearance (Hedgecock et al., 1983). Others were ced-3 and ced-4 (Ellis and Horvitz, 1986; Horvitz et al., 1983) and ced-9 (Hengartner et al., 1992), homologous to caspases, APAF-1 and bcl-2, respectively (Miura et al., 1993; Vaux et al., 1988; Yuan and Horvitz, 1990; Zou et al., 1997). These are clearly encoding key molecules in the actual mechanism of caspase-dependent cell death.

Altogether, in several (admittedly few) organisms in which a classical genetic approach to mechanism of cell death has been used, mostly signaling molecules have been identified. The apparent exception is *C. elegans*, an organism that interestingly is at the basis of our knowledge of the mechanism of caspase-dependent cell death. What could be the explanation for the fact that *C. elegans* may be the only organism [see, however, Thomas et al., (1998)] in which a classical genetic approach allowed the identification by mutagenesis of molecules involved in the mechanism of cell death per se? The explanation for the unusually high rate of mechanism mutations in *C. elegans* might reside in a combination of three factors. First, there is little or

no molecular redundancy in this mechanism in *C. elegans* (ced-3, ced-4, and ced-9 are unique), leading to a clear phenotype of no cell death in the corresponding mutants. Second, these mutations are well tolerated at the level of the whole organism: In *C. elegans*, cell death suppression by mutagenesis does not prevent apparently normal survival of the organism (Ellis and Horvitz, 1986), which may, however, suffer some nondevelopmental problems (Aballay and Ausubel, 2001). Third, and in contrast, in *C. elegans* most signalization mutants may severely affect cell death, but also development in a non-death-specific and detrimental manner (which is probably true for any organism). Altogether, in *C. elegans* most signalization mutants would be counterselected, while mechanism mutants would be at the same time detected and tolerated. These observations may have implications when devising strategies to obtain cell death mechanism mutants in other organisms. Interestingly, caspase-independent nonapoptotic cell death is also now studied in *C. elegans* through genetic methods (Chung et al., 2000; Xu et al., 2001).

## Conclusion

Our first campaign of mutagenesis in *Dictyostelium* yielded the DDM4 mutant, revealing the DlrA molecule and a novel mode of control of PKA expression. However, we were initially in search of molecules involved in cell death mechanism rather than cell death signaling. A critical analysis emphasized, first, a background problem leading to unexpected difficulties, encouraging us to pay more attention in further attempts to low background protocols, and, second, the possible predominance of mutants of signalization in this sort of approach. The latter conclusion leads us to consider for our second campaign another genetic approach, where downstream mutations would be selectable or at least easy to screen for. Many of the arguments discussed here would probably be valid for cell death mutant searches in other organisms.

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CHAPTER

4

# PROGRAMMED CELL DEATH IN *DROSOPHILA MELANOGASTER*

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As reviewed in previous chapters, programmed cell death (PCD) is necessary for the removal of unwanted and superfluous cells in tissue patterning during development and for homeostasis of the adult (see also Baehrecke, 2002). Although the study of cell death pathways in the worm *C. elegans* is well developed, and now much is known about the cell death machinery in mammals, *Drosophila melanogaster* is a relative newcomer to the field. *Drosophila* provides a system of intermediate complexity between worms and mammals in its PCD pathways, making it much more amenable for experimentation than the mammalian model systems. *Drosophila* shares many of the PCD components and pathways found in mammals, but has less redundancy, allowing easier dissection of function. The availability of the complete genomic sequence and the knowledge that most cell death components are conserved in *Drosophila* also has facilitated its acceptance as a key model system to study cell death regulation. Furthermore, *Drosophila* development is well characterized and genetically manipulable, thus being an ideal system in which to study developmentally programmed cell death. In addition, *Drosophila* is amenable to mutation analysis, transgenesis, genetic modifier screens, and RNAi-mediated gene ablation in vivo. Given

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these advantages, the study of cell death in *Drosophila* has progressed rapidly during the past ten years. In this chapter we review the current state of understanding of PCD regulation in the fly.

## Developmental PCD in *Drosophila*

Most of the PCD in *Drosophila* occurs during development and metamorphosis and is essential for the sculpting and refining of all developmental structures. The four stages of the *Drosophila* life cycle include the embryo, larva, pupa, and adult, and PCD can be seen throughout the fly life cycle. The first signs of cell death in the fly may be observed 6 hours after egg deposition (Abrams et al., 1993). Thereafter, apoptosis is vital during embryogenesis for cell fate specification during segmentation along the embryo anterior/posterior axis (Klingsensmith et al., 1989). Each segment comprises imaginal disc structures, which are ultimately responsible for giving rise to the principle structures and tissues in the adult organism (Rusconi et al., 2000). Extensive cell death can also be observed in cells of the central nervous system to provide spatial precision and organization of axons and the neural network. During *Drosophila* oogenesis, germline cell death is essential particularly to eliminate nurse cells once they have nourished developing oocytes, and for the generation of mature fertile eggs (Buszczak and Cooley, 2000). In the adult fly, apoptosis is required to further sculpt tissues, particularly the *Drosophila* retina, to provide a precisely ordered lattice network of ommatidia (Rusconi et al., 2000).

The most prominent PCD in the fly occurs during metamorphosis, where larval tissues undergo excessive reorganization to establish adult structures. The steroid hormone ecdysone induces the morphogenetic changes that occur throughout *Drosophila* development, and its major peak of activity occurs at the start of puparium formation, which triggers the start of metamorphosis (Jiang et al., 1997). Ecdysone is crucial for the controlled cell death of many larval tissues including midgut and salivary gland, during oogenesis and for the apoptosis of some muscle and neuronal cells in both larva and adult (Robinow et al., 1993; Buszczak and Cooley, 2000; Baehrecke, 2000). The details of ecdysone induced cell death in the fly are discussed in the "Ecdysone-mediated PCD during Metamorphosis" section below.

## The Core Cell Death Machinery in Fly

### Reaper, Hid, Grim, and Sickle

Most of the key components of the apoptotic machinery are conserved in the fly (Table 1). Homologues of the *Caenorhabditis elegans* proteins CED-3,

TABLE 1. Evolutionary Conservation of the Apoptotic Machinery. *Drosophila* contains most of the principal effector molecules of apoptosis that are highly conserved in both worm and mammals

| *C. elegans* | Mammals | *Drosophila* |
|---|---|---|
| Not present | Smac/Diablo, Htra2 | Rpr, Hid, Grim, Sickle |
| EGL-1 | BH3 only protein family | ? |
| CED-9 | Bcl-2 family | Debcl, Buffy |
| CED-4 | Apaf-1 | Dark |
| CED-3 | Caspase family | Dronc, Dredd, Strica, Drice, Dcp-1, Decay, Damm |
| BIR1, BIR2 | XIAP, cIAP1, cIAP2 | Diap1, Diap2 |
| Not present | FADD | dFADD |
| Not present | TNF | Eiger |
| Not present | TNFR | Wengen |

CED-4, and CED-9 have been found in *Drosophila*, but at present no EGL-1-like proteins have been described. The death machinery in *Drosophila* is of much higher complexity than the worm, and in accordance comprises additional proteins, many of which are functionally conserved in mammals.

The first genetic studies of apoptosis in *Drosophila* led to the identification of a deficiency [Df(3L) *H99*] that severely affected cell death during embryogenesis. This deficiency corresponds to a deletion of three essential apoptosis genes, *reaper* (*rpr*), *hid* (*head involution defect/wrinkled*), and *grim*, which are important mediators of developmental cell death (White et al., 1994; Chen et al., 1996). The expression of *rpr*, *hid*, and *grim* is upregulated during apoptosis, and each act in synchrony to induce the death of various embryonic tissues, including the CNS and death associated with the morphogenetic changes in the head region (Nassif et al., 1998; Wing et al., 1998). Most importantly though, *rpr* and *hid* expression is regulated by ecdysone and, in effect, is responsible for the metamorphic death of neurons, salivary gland, and midgut (Robinow et al., 1997; Jiang et al., 2000). Transcriptional upregulation of the *Drosophila* tumor suppressor gene *Dmp53* also directly induces the transcriptional activation of *rpr* in response to DNA damage (Nordstrom et al., 1996; Brodsky et al., 2000). The *rpr* gene contains a Dmp53 response element (*p53RE*), which is specifically induced in response to radiation, leading to enhanced *rpr* expression and cell death (Brodsky et al., 2000; Nordstrom and Abrams, 2000; Ollmann et al., 2000). Although these three genes are associated with the majority of embryonic PCD, they do not appear to be required for nurse cell death during oogenesis (Foley and Cooley, 1998). A recent study shows that *rpr* activity is also transcriptionally controlled by the homeobox protein Deformed, which maintains the boundary between the maxillary and mandibular head lobes by selective cell death (Lohmann

et al., 2002). Thus, Reaper appears to play a key function in developmental PCD in multiple tissues.

The expression of *hid* is not specifically confined to cells undergoing apoptosis, but is also detected in cells destined to survive, indicating that Hid may function in physiological events other than apoptosis (Grether et al., 1995). Hid activity is negatively regulated by the epidermal growth factor receptor (EGFR) signaling pathway (Kurada and White, 1998; Bergmann et al., 1998). Specifically, the mitogen-activated protein kinase (MAPK) downregulates the *hid* transcript and can also phosphorylate Hid protein, thereby ensuring suppression of Hid-induced cell death (Kurada and White, 1998). Recently characterized is a fourth gene that maps just outside of the *H99* region, named *sickle* (*skl*) (Christich et al., 2002; Wing et al., 2002). As with *rpr*, *hid*, and *grim*, the expression of *skl* is consistent with cell death during embryogenesis, but in contrast, *skl* cannot itself support all PCD in the embryo and is not expressed in all cells destined to die (Wing et al., 2002). Although the expression of *skl* alone does not appear to specify cell death, it is presumed that *skl* can act as an enhancer of PCD in cooperation with *rpr*, *hid*, and *grim* (Wing et al., 2002).

The only structural similarity between Rpr, Hid, Grim, and Sickle resides in a short N-terminal sequence, referred to as the RHG motif (Wing et al., 1998, 2002; Christich et al., 2002). Grim, Rpr, and Sickle also contain a GH3 motif, an internal 15 amino acid amphipathic α-helical domain, which in contrast is essential for the pro-apoptotic activity of these proteins (Claveria et al., 2002). Furthermore, the GH3 motif in Grim is required for its mitochondrial targeting and subsequent activation of caspases (Claveria et al., 2002). These RHG-containing proteins are able to induce cell death by inactivating the *Drosophila* inhibitor of apoptosis proteins (Diap1 and Diap2; see section entitled "*Drosophila* Inhibitor of Apoptosis Proteins"). In effect, Rpr, Hid, and Grim are able to interact with Diap1 in an RHG-dependent manner to promote Diap1 ubiquitination and degradation (Wang et al., 1999; Goyal et al., 2000; reviewed in Martin, 2002). The expression of an amino-terminal RHG truncation of Grim potently induces apoptosis that is not inhibited by Diap1, and cannot be suppressed by caspase inhibition (Wing et al., 2001). These findings imply that Grim can function to promote cell death independent of caspases. Grim and Rpr are also able to induce a general inhibition of translation, which correspondingly decreases the levels of Diap1 protein and thereby allows the apoptosis pathway to proceed (Holley et al., 2002; Ryoo et al., 2002). There are no structural homologues of Rpr, Hid, and Grim in mammals. However, two putative functional homologues containing an RHG-like motif named Smac/Diablo and Htra2 have been identified based on their ability to interact with mammalian IAPs (Du et al., 2000; Verhagen et al., 2000, Suzuki et al., 2001; Hedge et al., 2002; Martins et al., 2002; Van Loo et al., 2002; Verhagen et al., 2002). Both these proteins can inactivate IAP-like proteins to facilitate caspase activation in mammalian cells (Du et al., 2000; Verhagen et al., 2000; Martins, 2002).

## Bcl-2 Homologues

In *C. elegans* there are two Bcl-2-like family members, a pro-apoptotic BH3-only protein EGL-1 and a prosurvival Bcl-2-like protein CED-9 (see Chapter 11). In mammals, the Bcl-2 family of proteins comprises at least 20 pro-apoptotic and pro-survival members, which interact with each other to determine cell fate (reviewed in Baliga and Kumar, 2001). The regulation of apoptosis by Bcl-2-like proteins in mammals is a highly complex process and their precise course of action is still unclear. In *Drosophila*, there are two Bcl-2-like proteins, both of which are closely related to mammalian Bax-like proteins. One of these, Debcl/dBorg-1/d-Rob-1/dBok, acts as a pro-apoptotic member (Brachmann et al., 2000; Colussi et al., 2000; Igaki et al., 2000; Zhang et al., 2000; reviewed by Chen and Abrams, 2000). The other Bcl-2-like member, Buffy, appears to act as an antiapoptotic protein (Quinn et al., 2003). Both Debcl and Buffy contain three BH domains (BH1, BH2, BH3) and a carboxyl-terminal membrane anchor that localizes to mitochondrial or ER membranes. Surprisingly, unlike its mammalian pro-survival Bcl-2 counterparts, Buffy lacks a BH4 domain and, like Debcl, it shares the greatest similarity with pro-apoptotic Bok. Debcl induces cell death when overexpressed in various *Drosophila* tissues, and ablation of *debcl* by RNAi significantly reduces the level of embryonic PCD, emphasizing the importance of Debcl in developmental cell death (Igaki et al., 2000; Brachmann et al., 2000; Colussi et al., 2000). Genetic studies have shown that Debcl promotes cell death through CED-4-like protein Dark, in a caspase-dependent fashion (Colussi et al., 2000). Currently, no pro-apoptotic EGL-1-like protein has been characterized in the fly.

## *Drosophila* Inhibitor of Apoptosis Proteins

Inhibitors of apoptosis (IAP) proteins serve to regulate apoptosis through the direct binding and inhibition of caspases (reviewed in Goyal et al., 2001). Similar to their viral counterparts, the *Drosophila* IAPs, Diap1 and Diap2, contain two amino-terminal BIRs (baculovirus inhibitor of apoptosis repeat) that mediate binding to caspases, and a carboxy-terminal RING finger domain that has E3 ubiquitin-ligase activity (Hay et al., 1995; Yang et al., 2000). Diap1 and Diap2 were among the first cellular IAPs to be isolated as potent suppressors of death induced by *rpr*, *hid*, and *grim* (Hay et al., 1995; reviewed in Hay, 2000; Goyal et al., 2000). Early studies characterized a loss-of-function mutation in *diap1*/*thread* in a genetic screen that strongly enhanced cell death induced by ectopic expression of *rpr*, *hid*, and *grim* in the developing fly eye (Hay et al., 1995). In fact, loss of *diap1*/*thread* function is embryonic lethal and embryos display increased caspase activity, which suggests that Diap1 acts to suppress apoptosis through the inhibition of caspases (Wang et al., 1999). It has now been established that Diap1 functions by directly interacting with the caspases Dcp-1, Drice, and Dronc to inhibit

their processing and activation (Meier et al., 2000; Hawkins et al., 1999; Kaiser et al., 1998; Wang et al., 1999; Goyal et al., 2000; Quinn et al., 2000; Muro et al., 2002). During apoptosis, Rpr, Hid, and Grim are able to directly interact with Diap1, thereby disrupting the Diap1–caspase interaction (Wang et al., 1999; Goyal et al., 2000). The removal of Diap1 inhibition occurs concurrently with Dark-dependent activation of caspases (Igaki et al., 2002b; Rodriguez et al., 2002). The RHG domain of Rpr, Hid, and Grim was initially thought to be essential for the interaction with the BIR2 domain of Diap1; however, deletion mutants of this domain are still able to promote cell death and complex with Diap1 (Chen et al., 1996; Vucic et al., 1998; Wing et al., 1998; Goyal et al., 2000). Nevertheless, it is clear that binding of these RHG-proteins to Diap1 stimulates Diap1 degradation through autoubiquitination (Hays et al., 2002; Holley et al., 2002; Yoo et al., 2002). Diap1 degradation is further enhanced by the E2 ubiquitin conjugase-related protein, Morgue, that acts in synergy with Rpr, Hid, and Grim to regulate Diap1 levels and promote apoptosis (Hays et al., 2002). Although controversial, Diap1 was recently shown to promote degradation of Dronc, which would suggest that Diap1 does not merely sequester and silence caspase activity, but can target caspases for ubiquitination and proteosomal degradation (Wilson et al., 2002). The precise function of Diap2 in cell death inhibition is obscure. Diap2 is able to inhibit Dronc-induced apoptosis in the fly eye, but cannot physically interact with Dronc (Quinn et al., 2000). In fact, the only caspase shown to physically associate with Diap2 is Strica (Doumanis et al., 2001). Diap2 can interact with the *Drosophila* Decapentaplegic (Dpp) Type-I receptor, thick veins (Tkv) (Oeda et al., 1998), which implicates a possible role for Diap2 in apoptosis regulation via the Jun N-terminal kinase (JNK) signaling pathway. Additional *Drosophila* IAP-like proteins, Deterin and dBRUCE, have since been characterized in the fly, but their exact role in cell death is not yet understood (Jones et al., 2000; Vernooy et al., 2000).

## Caspases, Adaptors, and Caspase Activation

The *Drosophila* caspase family consists of seven members, including Dcp-1, Dredd/Dcp-2, Drice, Dronc, Decay, Strica, and Damm (Song et al., 1997; Fraser and Evan, 1997; Chen et al., 1998; Dorstyn et al., 1999a, b; Doumanis et al., 2001; Harvey et al., 2001; reviewed by Kumar and Doumanis 2000). Three of these, Dredd, Dronc, and Strica, contain long amino-terminal pro-domains, while the rest have short or absent pro-domains and thereby are likely to be downstream caspases (Kumar and Doumanis, 2000). As in mammals, the long amino-terminal pro-domains in Dronc and Dredd contain specific protein–protein interaction motifs, which are likely to be required for recruitment of caspases to adaptor molecules to promote their autocatalytic activation. Dredd contains two DEDs (death effector domains) in its prodomain region, which is similar to mammalian caspase-8 and -10,

the mediators of the death receptor signaling pathways. Dronc has a CARD (caspase recruitment domain) that can mediate its interaction with the CARD in Dark (Dorstyn et al., 1999a). Interestingly, Strica contains a unique amino-terminal Ser/Thr-rich domain of unknown function, which is devoid of any known protein–protein interaction motifs (Doumanis et al., 2001).

The first *Drosophila* caspase identified was Dcp-1, which shares the highest homology with mammalian caspase-3 and -7 (Song et al., 1997). The importance of Dcp-1 in developmental PCD has been illustrated by *dcp-1* null mutations, which results in third instar larval lethality (Song et al., 1997). Surviving larvae have a normal central nervous system but exhibit melanotic tumors and interestingly lack imaginal discs and gonads (Song et al., 1997). A loss of function mutation in *dcp-1* causes defects in somatic and germ cell development due to the deficient transfer of nurse cell constituents to nourish developing oocytes, so consequently, female flies lacking *dcp-1* are sterile (McCall and Steller, 1998). Consistent with its role as a caspase effector, transgenic expression studies have established that Dcp-1 can promote cell death in the *Drosophila* eye and that Rpr and Grim are able to activate Dcp-1 in the same death pathway (Song et al., 2000).

Similarly to Dcp-1, Drice shares the highest homology with caspase-3 and -6 (Fraser and Evan, 1997). As no *drice* mutants are currently available, the importance of Drice in apoptosis has been established by studies in the *Drosophila* S2 cell line. Drice is rapidly processed and activated in apoptotic cells, and the depletion of Drice significantly reduces caspase activity and apoptosis (Fraser and Evan, 1997; Fraser et al., 1997). Drice is able to cleave *Drosophila* DNase (CAD) and its inhibitor (ICAD), resulting in DNA fragmentation during cell death (Yokoyama et al., 2000).

Dredd/Dcp-2 shares the highest degree of homology with mammalian caspase-8 (Chen et al., 1998). As mentioned previously, Dredd contains two amino-terminal DEDs that are able to interact with *Drosophila* Fas-associated death domain protein, FADD, but its role in a Fas-like death receptor pathway in the fly has not been documented (Hu and Yang, 2000). Dredd is processed and activated by Rpr, Hid, and Grim, and the loss of a *dredd* allele affects cell death induced by *rpr* and *grim*, indicating that Dredd acts to induce cell death downstream of these apoptotic inducers (Chen et al., 1998). In addition to its role in PCD, Dredd was recently found to be a regulator of the *Drosophila* immune response (Elrod-Erikson et al., 2000; Leulier et al., 2000). Mutations in *dredd* resulted in impaired inducibility of antibacterial genes and high susceptibility to bacterial infection. Dredd appears to function to activate the NFκB-like protein, Relish, which in turn acts to transcriptionally activate various antimicrobial peptide genes (Stoven et al., 2000).

Dronc is the only *Drosophila* caspase that contains a CARD and is likely to be the CED-3/caspase-2/-9 homologue in the fly (Dorstyn et al., 1999a). Interestingly, *dronc* mRNA expression is upregulated in late third instar

larval salivary glands and midgut in response to ecdysone, and recent data suggest that Dronc is an essential mediator of ecdysone-regulated cell death (Dorstyn et al., 1999a; Lee et al., 2000; Lee and Baehrecke, 2001; Cakouros et al., 2002). Dronc overexpression causes cell death in transgenic flies, and its ablation by siRNA results in a block in embryonic PCD (Meier et al., 2000; Hawkins et al., 2000; Quinn et al., 2000). Dronc is required for cell death mediated by Rpr, Hid, and Grim (Quinn et al., 2000).

The remaining three caspases, Damm, Decay, and Strica, have not been well characterized, and their specific function in cell death is not known at present (Dorstyn et al., 1999b; Doumanis et al., 2001; Harvey et al., 2001). Ectopic overexpression of *damm* in the *Drosophila* eye results in a slight rough eye phenotype, and the expression of *damm* sensitizes cells to apoptosis (Harvey et al., 2001). Interestingly, Damm is suggested to function downstream of Hid in apoptosis (Harvey et al., 2001).

In mammals, the CED-4 homologue, Apaf-1, is an essential component of the "mitochondrial death pathway" for the activation of casapse-9 (Zou et al., 1999). In *Drosophila* there is a single CED-4-like protein Dark/Dapaf-1/Hac-1 (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999). Like CED-4/Apaf-1, Dark is an essential mediator of developmental PCD, and although hypomorphic *dark* mutants are viable, they exhibit many cell death defects, including melanotic tumors, neuronal hyperplasia, defective wing development, and extra cells in the eye (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999). In addition, *dark* mutant embryos exhibit a general decrease in the number of apoptotic cells and reduced caspase activity, indicating that like CED-4/Apaf-1, Dark-mediated cell death depends on the activation of caspases (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999; Quinn et al., 2000). Recent data show that Dark mediates the activation of Dronc (Quinn et al., 2000; Dorstyn et al., 2002). Like Apaf-1, Dark contains a large C-terminal WD40 region that can also complex in vitro with cytochrome c. However, cytochrome c is not released from mitochondria during fly cell death, and RNAi-mediated knockdown of cytochrome c in *Drosophila* cells has no apparent effect on caspase activation (Dorstyn et al., 2002; Zimmermann et al., 2002). Thus, it is likely that despite the similarities between Apaf-1 and Dark, Dark-mediated Dronc activation may not require cytochrome c.

## The TNF Pathway in the Fly

Until recently, an extrinsic receptor-mediated death pathway had not been documented in *Drosophila*. This was partly due to the fact that the *Drosophila* genomic sequence did not uncover any proteins with significant homology to mammalian death domain (DD) or DED containing receptors (Vernooy et al., 2000; Aravind et al., 2001). As mentioned above, although there is a

FADD-like molecule in *Drosophila* (dFADD), it does not appear to be involved in a death receptor signaling pathway. The DD-containing proteins in *Drosophila* largely function in the innate immune response (Imler and Hoffman, 2001; Georgel et al., 2001). Given that no other DD-containing protein is known to interact with dFADD, it appears that dFADD may only function to activate DREDD primarily during innate immune response. However, further studies are required to fully establish the function of dFADD.

The identification of *Drosophila* tumor necrosis factor receptor associated factors (dTRAF), which can activate NFκB, was the first indication that a tumor necrosis factor receptor (TNFR) signaling pathway may exist in the fly (Liu et al., 1999; Zapata et al., 2000). Recently, a TNFR-like molecule Wengen and its ligand Eiger, were characterized in *Drosophila* (Igaki et al., 2002a; Kanda et al., 2002; Moreno et al., 2002). Initial studies with Wengen demonstrated that it is a transmembrane protein comprising the conserved TNFR homology domain that acts as a functional receptor for the TNF ligand Eiger (Kanda et al., 2002). Eiger is also a transmembrane protein with a carboxy-terminal TNF homology domain and appears to activate cell death in association with dTRAF, through the JNK pathway (Liu et al., 1999; Igaki et al., 2002a; Moreno et al., 2002). Interestingly, Eiger-mediated cell death does not require dFADD or Dredd, but rather depends on the activity of Dark and Dronc (Moreno et al., 2002) and is inhibited by Diap1 (Igaki et al., 2002a; Moreno et al., 2002) (Fig. 1). Furthermore, the expression of Eiger induces transcriptional upregulation of *hid* and to a lesser extent *rpr* (Moreno et al., 2002). Together, these results provide a link between the extrinsic death receptor pathway and the intrinsic pro-apoptotic signaling components. JNK signaling has previously been associated with epithelial morphogenesis and segment polarity during embryonic development, but its precise mechanism of signaling is poorly understood in *Drosophila* (Noselli and Agnaas, 1999). Although *wengen* is expressed at all stages of *Drosophila* development, the expression of *eiger* is predominant in the nervous system, indicating that Eiger may be involved with normal development of the CNS (Igaki et al., 2002a; Kanda et al., 2002). Moreover, it is possible that additional unidentified TNF-like family members in the fly function to activate the cell death program in other tissues.

## Ecdysone-Mediated PCD During Metamorphosis

In vertebrates steroid hormones play a crucial role in regulating cell proliferation and cell death, but the signaling mechanisms underlying their action remain poorly understood. PCD during *Drosophila* metamorphosis is primarily regulated by a single steroid hormone 20-hydroxyecdysone (commonly called ecdysone), and recent studies in the fly have provided much

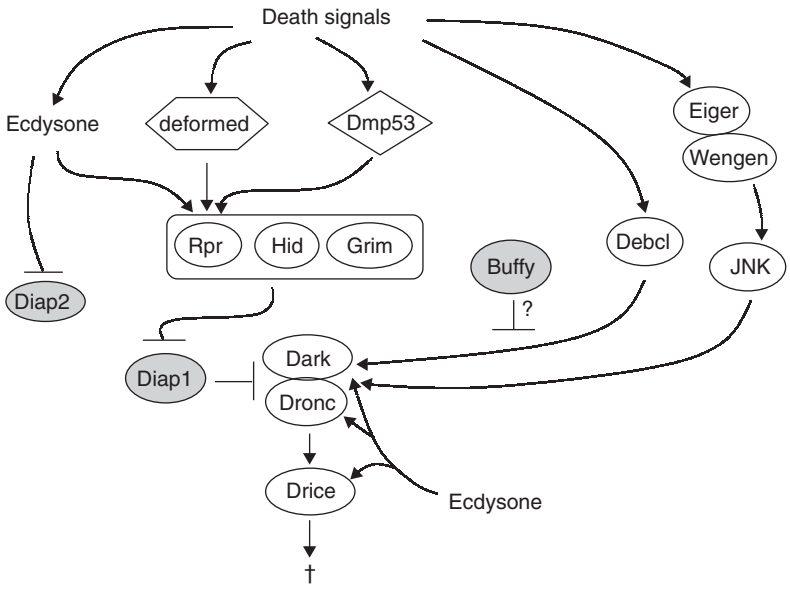


FIGURE 1. Cell death pathways in *Drosophila*. The main components of the fly cell death machinery and their known and potential interactions are shown. Antiapoptotic proteins are shaded. The activation of caspases can occur through inhibition of DIAP1 by Rpr, Grim, and Hid or through Eiger–Wengen receptor-mediated JNK signaling pathways.

insight into the mechanisms of steroid-mediated cell death (Baehrecke, 2000). At the onset of metamorphosis, a large increase in ecdysone levels triggers the cell death of larval midgut and anterior muscle, and then a second peak 12 hours postpuparium formation induces the PCD of larval salivary glands (Jiang et al., 1997; Lee and Baehrecke, 2001). Ecdysone mediates its effects through a heterodimeric receptor complex, which comprises the nuclear hormone receptor family members, ecdysone receptor (EcR), and ultraspiracle (Usp) (Thomas et al., 1993; Hall and Thummel, 1998; Baehrecke, 2000). This ecdysone receptor complex is able to directly activate the transcription of several early genes, including *Broad-complex* (*BR-C*), *E74A*, and *E75*. Each of these genes encodes transcription factors that regulate transcription of late genes in a tissue and stage-specific manner, to induce cell differentiation and cell death (Thummel, 1996; Baehrecke, 2000). The initial peak of ecdysone at the end of the third instar larval stage induces activation of *BR-C*, *E74*, and *E75* genes, which in turn regulate transcription of glue genes in the salivary gland (Lee and Baehrecke, 2001). The factors responsible for specifying the initiation of PCD in larval midgut remain obscure, but it is clear that the expression of both *BR-C* and *E93* is crucial for midgut cell death

(Lee et al., 2002a). In fact, ecdysone induction of *BR-C* and *E93* leads to an increase in transcription of the apoptosis genes *rpr*, *hid*, *dark*, and *dronc*, which ultimately cause destruction of the larval midgut (Lee et al., 2002a). A second peak in ecdysone titer occurs prior to salivary gland histolysis and initiates transcription of *BR-C*, *E74*, *E75*, and *E93* early genes. The competence factor *βFTZ-F1* is upregulated just prior to the ecdysone peak and is necessary for the reinduction of *BR-C*, *E74*, *E75*, and *E93* genes (Baehrecke, 2000).

The *E93* gene is transcribed in a stage-specific manner, and while the other transcription factors act to mediate a variety of regulatory responses, *E93* appears to function specifically during the cell death of larval tissues (Lee et al., 2002a, b). *βFTZ-F1* functions as a pupal-specific factor, and its expression is crucial for DNA fragmentation and the destruction of larval salivary glands (Lee et al., 2002b). A loss-of-function mutation in *βFTZ-F1* dramatically reduces the transcription of *BR-C*, *E74*, and *E93* genes (Broadus et al., 1999). In addition, the ectopic expression of *βFTZ-F1* is sufficient to induce early transcription of the cell death genes *rpr*, *hid*, *dark*, *dronc*, and *crq*, thereby making them able to prematurely initiate salivary gland death (Lee et al., 2002b). *E75* suppresses the *βFTZ-F1* mediated upregulation of *diap2* in late prepupal salivary glands, thus allowing PCD to proceed (Jiang et al., 2000). Furthermore, *E93* is an important regulator of cell death mediated by *βFTZ-F1* expression (Lee et al., 2002b). While *BR-C* and *E74A* are essential for the maximal induction of *rpr* and *hid* in salivary glands, *E93* is vital for the proper transcriptional upregulation of *dark*, *dronc*, and *crq* (Jiang et al., 2000). Mutations in *E93* also affect transcription of both *BR-C* and *E74A* and consequently result in decreased levels of *rpr* and *hid*, indicating that *E93* is the principal effector of salivary gland PCD (Lee and Baehrecke, 2000; Lee et al., 2000). In addition, *BR-C* can directly regulate *dronc* transcription (Cakouros et al., 2002). Recent data suggest that *drice* is also controlled by ecdysone in salivary glands (Kilpatrick and Kumar, unpublished), suggesting that upregulation of the death effectors such as Dronc, Dark, and Drice, and a downregulation of PCD inhibitors such as Diap1 may regulate large-scale PCD during metamorphosis.

## Perspectives

*Drosophila* has thus far proved to be an important tool for the study of PCD and has provided useful information for understanding the functions of individual components of the apoptotic signaling pathways in vivo. Although most of the core cell death machinery in the fly is similar to that in mammals, there are several unique features of *Drosophila* cell death. For example, while Reaper, Hid, and Grim play a very significant initiator role in *Drosophila* cell death, the mammalian functional homologues of these proteins, Diablo and

Htra2, have a relatively minor role in promoting downstream caspase activation. The role of IAPs, particularly Diap1, is central in the regulation of cell survival in the fly, whereas knockout studies suggest that IAPs serve more of a side role in mammals (Hay, 2000). Another unresolved issue is the role of cytochrome c in caspase activation in *Drosophila*. Although cytochrome c has not been shown to play any role in CED-4-mediated CED-3 activation in *C. elegans*, in mammals it is crucial for the formation of the "Apaf-1 apoptosome" that recruits and activates caspase-9 and subsequently caspase-3 (Zou et al., 1999; Cain et al., 2002). A lack of cytochrome c release from mitochondria during apoptosis of fly cells indicates that cytochrome c may not be required for Dark-mediated Dronc activation (Dorstyn et al., 2002). Significantly, Dark contains multiple WD40 repeats similar to those found in Apaf-1 (but not in CED-4), and has the ability to bind cytochrome c in vitro (Kanuka et al., 1999). Thus, the key question that remains to be addressed is whether in the absence of cytochrome c release, Dark binds a yet unknown protein via its WD40 repeats, which mediates its oligomerization. If such a protein exists, then we will need to establish where it is localized and what promotes its binding to Dark during cell death.

Perhaps the most unexpected feature of the core death machinery in *Drosophila* is the relatively minor role of Bcl-2 family members. There have been no BH3-only proteins discovered in the fly so far, but this may simply be because of a low degree of BH3 sequence conservation, making it difficult to identify them based on sequence homology. More interesting, however, is the observation that both Bcl-2 homologues in the fly are related to pro-death Bax-like protein Bok in mammals, even though one, Buffy, may function as a pro-survival protein. This is surprising as CED-9, the only Bcl-2 homologue in *C. elegans*, is a pro-survival protein with all four BH domains (BH1–BH4) that are characteristic of the mammalian pro-survival Bcl-2 family members (reviewed in Baliga and Kumar, 2002). While CED-9 in *C. elegans* is essential for the survival of all cells, it remains to be determined whether Buffy is a universal cell survival molecule, or its function is required only in some cell types. The exact physiological function of Debcl in specific cell death pathways is also far from understood. Many of these questions await creation/isolation of appropriate fly mutants and their characterization to fully understand the role of various cell death proteins in developmental PCD.

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CHAPTER

5

# CELL DEATH IN PLANT DEVELOPMENT AND DEFENSE

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Programmed cell death (PCD) is emerging as a fundamental concept in modern biology. It plays a vital role in almost all known organisms from unicellular bacteria to complex multicellular organisms such as animals. Within these two evolutionary extremes, plants may serve as an excellent example of how a relatively simple multicellular organism uses PCD for many of its functions. The study of PCD in plants may therefore provide a link between PCD in unicellular organism and PCD in more complex multicellular organisms from different kingdoms.

Unlike animal cells, plant cells contain rigid cell walls composed mainly of cellulose, a large vacuole that participates in many chemical and biochemical processes, and a specialized organelle conducting photosynthesis, that is, the chloroplast. Plants do not have an immune system similar to animals, but are capable of mounting a large array of defenses, including PCD, when attacked by pathogens. In addition, the development of plants is solely determined by cell division and not by a combination of cell division and cell migration, as in animals. Despite these differences, plants, much like animals, require PCD for their proper development and response to different environmental stimuli (Greenberg, 1996; Dangl et al., 1996; Lam et al., 2000; Beers and McDowell, 2001).

When considering PCD in plants, it is important to remember that although PCD in plants is similar in its conceptual and functional definitions to PCD in animals, in many characteristic and mechanistic aspects PCD in

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plants is different from apoptosis in animals (Mittler, 1998). For example, the lack of an immune system eliminates the need for the formation of apoptotic bodies and the "clean" removal of cells, because an inflammatory response is not likely to occur when the content of a plant cell is spilled. In addition, in most examples of PCD in plants the cell wall remains intact, making the trafficking of apoptotic bodies from one cell to the other, or the engulfment of a dying cell by neighboring cells, unlikely. Despite these differences, the genome of plants appears to encode for a large number of PCD pathways activated during development, differentiation, and the defense of plants against different biotic and abiotic insults.

## PCD during Development

From embryogenesis and seed germination to fertilization, the life cycle of a plant is marked by dramatic morphological evolution on the organ and whole plant levels. The biochemical, molecular, and cellular events that orchestrate the ontogeny of new cells at the meristems which give rise to morphologically distinct organs, and those that activate distinct biochemical pathways to support specialized functions of different tissue and cell types have been extensively studied. Similarly important but often less commanding of our attention are cell dedifferentiation, degeneration, and death processes that permit the plasticity which is associated with plant development. Every stage of plant development is marked by PCD events. Examples include selective cell death during embryogenesis, xylem (water-conducting system) differentiation, leaf and flower petal senescence. Development of the reproductive organs and the reproductive processes of pollination and fertilization are a progression of PCD processes in meristematic cells to terminally differentiated cell types within the male and female organs as well as in the gametophytes (pollen and embryo sac) themselves (Fig. 1).

### Leaf Senescence

In vegetative development, leaf senescence provides a most vivid example of the consequence of PCD in plants (Quirino et al., 2000). Leaf senescence is apparently a genetically regulated process involving active gene activity (Oh et al., 1997; Nam, 1997). It is also regulated by both intracellular signals such as the phytohormone cytokinins and ethylene and environmental signals such as light (Park et al., 1998). Visible loss of chlorophyll in senescing leaves is actually preceded by structural and biochemical deterioration of the chloroplasts. Increased cysteine proteases, nucleases, stress-related enzymes, and defense-related proteins are associated with leaf senescence

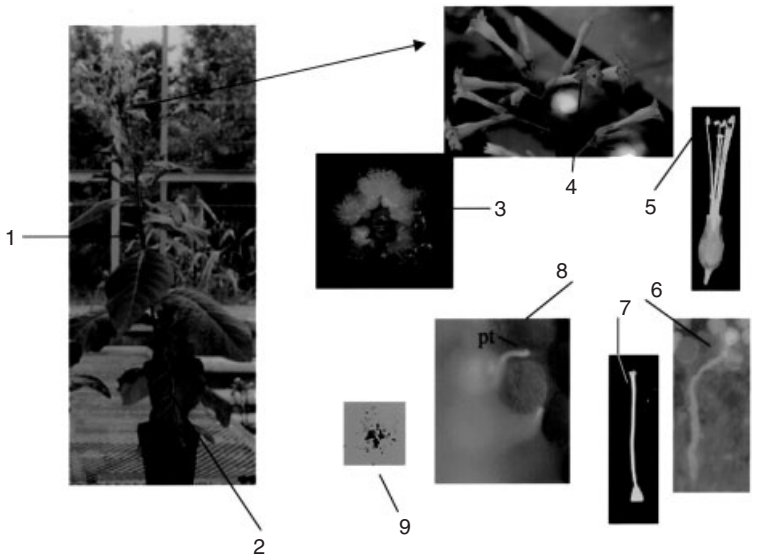


FIGURE 1. Major PCD events during plant development: (1) Tracheary element differentiation in the formation of water-conducting vessels. (2) Leaf senescence. (3) Sex determination in unisexual flowers; picture shows a very young floral bud with developing floral organ primordia. The most internal organ is the emerging gynoecium. The male initials are not visible here. (4) Petal senescence. (5) Tapetum cell death in pollen development; picture shows several anthers filled with mature pollen. (6) Pollen cell death in incompatible pollination; picture shows an arrested pollen tube. (7) Female tissue death in mature and pollinated female organ; pollen tubes grow within this organ. (8) Cell death in the guided entrance of pollen tubes (pt) into the female chamber, the embryo sac. (9) Cell death in seed development and germination. [Illustrations are not to scale. Some of the PCD phenomena are not universal to all plants.]

(see Quirino et al., 2000). Mitochondria apparently remain active until the final stages of leaf senescence, probably to provide the energy needed for resource mobilization. The ensuing oxidative damages as the result of an aging- or stress-induced decline in photosynthetic activity might signal other biochemical changes accompanying senescence in similar capacity as mitochondria are involved in PCD in other cell systems (Bleeker and Patternson, 1997; Susin et al., 1998; Jones, 2000; Quirino et al., 2000). Although degradation of the photosynthetic membrane system is a biochemical and structural hallmark of leaf senescence, the senescent program at least transiently maintains or probably even induces the pigments (carotenoids) and membrane components that constitute the chromoplasts, especially in species that display a period of bright color display before abscission. Except for a more

exaggerated chromoplast developmental pathway, tomato fruit ripening may be considered an analogous senescent event to leaf senescence in terms of the involvement of changes in plastid pigment contents. The light-harvesting membrane and carotenoid-binding membrane systems clearly coexist in the plastids of a ripening, or scenescing, tomato (Cheung et al., 1993). Therefore, even in dying, plant cells are programmed for a last episode of activity.

## Xylem Differentiation

Less obvious to the casual observer, the differentiation of tracheary elements (TE) in developing plants is the result of terminal differentiation involving PCD and the dismantling of progenitor cells to form the xylem vessels that transmit water from roots to shoot (Fukuda, 2000). Understanding the in situ events of TE differentiation has been much aided by the availability of an in vitro traecheid differentiation system from cultured *Zinnia* mesophyll cells. In response to proper auxin and cytokinin conditions, these mesophyll cells initially dedifferentiate, a state that could be considered analogous to conditions at the meristem where cell fate is still plastic. These dedifferentiated cells progress in two stages, II and III, during which cellular and biochemical changes, cell structural changes such as wall thickening and lignification, and ultimately cell death occur, mimicking the *in planta* process (Fukuda, 2000). Studies made of the *Zinnia* system have allowed the predetermined TE precursor cells in stage II to be distinguished from those that have undergone the final differentiation steps in stage III. This allowed the identification of several critical cellular, molecular, and ionic factors involved in TE differentiation (e.g., Roberts and Haigler, 1990; Kobayashi and Fukuda, 1994; Yamamoto et al., 1997; Groover et al., 1997; Woffenden et al., 1998; Groover and Jones, 1999; Fukuda, 2000; Yu et al., 2002). Vacuole collapse and the cessation of cytoplasmic streaming are the earliest signs of organelle degradation, followed by the degeneration of other membranous structures, including the mitochondria whose inner membrane depolarizes, and ultimately autolysis. Chromatin degradation, as revealed by TUNEL assay, is a relatively late event in TE differentiation, believed to be promoted by nucleases released upon vacuole collapse. Cysteine, serine proteases, and components of the ubiquitin-regulated proteolytic pathways are apparently involved in the PCD, leading to TE formation. Besides auxin and cytokinins being involved in triggering the initiation of TE differentiation, brassinosteroid, which apparently is synthesized in stage II cells, plays a critical role in promoting the transition into stage III events. Influx of $Ca^{2+}$ precedes and is necessary for entrance into stage III, and transient increases in calmodulin and calmodulin binding proteins also accompany this transition, suggesting the involvement of this ubiquitous regulator in TE-PCD. Although cytochrome c release accompanies mitochondria degeneration, it is not suf-

ficient to trigger PCD in these cells (Yu et al., 2002), suggesting the mitochondria involvement in TE-PCD occurs through a mechanism distinct from that of mammalian cell death (Earnshaw, 1999).

## Petal Senescence

Nature's most unforgiving way of illustrating mortality among creatures is probably the short life-time of flowers. By the time a flower opens, most cells have progressed to considerably advanced stages of declining activities. Studies in the highly synchronized life cycle of the daylily flower, which spans a 24-hour period and is most likely hardwired to an internal program, provided the most thorough correlation of biochemical and physiological events during the cell death process that occurs in senescing petals (Rubinstein, 2000). In general, increasingly oxidative conditions and loss of cell membrane permeability are early events in petal PCD. Gene expression and enzyme activity patterns reflect the enzymatic needs to accomplish degenerative cellular conditions during petal senescence. Proteases, DNases RNases, sugar hydrolases, and enzymes related to lipid metabolism are all upregulated. The senescing pathway in petals most likely shares significant similarity with other senescing plant cell systems. For example, common senescence-associated RNases could be found in both leaves and petals (Taylor et al., 1993; Lers et al., 1998), and proteases common to those induced in other senescing systems have been observed to be upregulated in petals (Buchanan-Wallaston, 1997; Stephenson and Rubistein, 1998; Courtney et al., 1994; Panavas et al., 1999).

While petal development per se may be viewed already as the initiation of senescence, abrupt aging and death in the petals of many flowers are induced only after pollination (Van Doorn, 1997). For instance, pollinated tobacco flowers show visible signs of cell death within 24 hours after pollination and abscise in about 3 days after anthesis (and pollination). On the other hand, unpollinated flowers remain vibrant for at least 3 days longer than pollinated ones and abscission occurs 1 week after anthesis. In the orchid *Phalaenopsis*, flowers are in bloom for months but pollination induces death within a day (Halevy, 1998). This would seem a reasonable strategy for flowers to adopt to prolong their attraction to pollination agents. Once the mission is accomplished, they quickly alter to a mode in which defense and resource mobilization should be the most important goal for preservation of the next generation. Floral senescence will minimize opportunistic infestation and recycle cellular resources to the developing fruits and seeds. Pollination-induced petal senescence probably invokes similar mechanisms as developmentally regulated senescence. For instance, increase levels of nucleases, membrane disorganization, and DNA fragmentation are all associated with pollination-induced senescence in petunia and these processes are enhanced by $Ca^{2+}$ (Xu and Hanson, 2000).

## Reproductive Development

Sex Determination. Cell death occurs early in reproductive development in the selective abortion of primordia for one or the other sexual organ in some unisexual flowers (Wu and Cheung, 1998, 2000). For example, in maize, the male flowers are segregated to the plant apex, whereas the female flowers develop in the ears borne on the lower part of the stem. However, early in development, the flowers of maize are hermaphroditic, producing primordia for both stamens and carpels in all the floral meristems. Early in development, gynoecial initials are aborted in the male-destined flowers; slightly later in development, male differentiation is arrested in the female-destined flowers, resulting in separate male and female flowers on the same plant (Irish and Nelson, 1989). In both cases, cellular vacuolization and the loss of organelle and cytoplasmic integrity precede primordial abortion or arrest (Cheng et al., 1983). Genes defective in gibberellic acid biosynthesis induce masculinized female flowers (Bensen et al., 1995; Winkler and Helentjaris, 1995), indicating a critical role for this hormone in suppressing male differentiation in female-destined flowers. Characterization of mutants with feminized male flowers, *tasselseed2*, led to the identification of a gene with homology with steroid dehydrogenases (De Long et al., 1993). The Tasselseed2 mRNA is found in the gynoecial primordium before its abortion in developing male-destined florets, suggesting a role for a steroid-related compound in arresting female development in these flowers.

Anther and Pollen Development. In a later stage of the reproduction phase that involves the development of the male gametophyte (pollen) within the anther, cell degeneration and death in different anther tissues are important for pollen maturation and dispersal. Pollen grains are covered with a coat of proteinaceous, lipoidal, and carotenoid-containing materials that are largely derived from cells in the tapetal layers, which line the chamber in which pollen develops. Tapetal cells secrete nutritive materials during pollen development and increasingly more hydrolytic enzymes against cell wall polymers to release individual pollen grains from a tetrad arrangement as pollen matures. Mature tapetal cells lack a well-developed primary cell wall and are packed with secretory organelles on the surface exposed toward the developing pollen. They begin to degenerate even before pollen fully mature. Tapetal cell deterioration is marked by cell shrinkage, polarization of cytoplasmic materials, vacuolation, and thinning of cell walls. On the cytological levels, tapetal cell degradation shows condensation of chromatin, swelling of the endoplasmic reticulum, and the persistence of mitochondria (Papini et al., 1999), typical of PCD. DNA fragmentation may also be associated with this process (Wang et al., 1998). Ultimately, these cells rupture and degeneration of the entire tapetum occurs (Chapman, 1987;

Bedinger, 1992). Through their death, the tapetal cells further contribute to the extracellular architecture of the pollen grains and provide their surface with adhesive and signaling molecules important for interactions with the female tissues. Deficiency in the pollen coating, defects in tapetal secreted enzymes, arrest in tapetal development, or precocious tapetal deterioration can all result in male sterility (Wu and Cheung, 2000). At maturity, pollen grains need to be exposed and dispersed from the anther to achieve pollination. This again requires the breakdown of specific anther cells that leads to anther rupture, exposing the pollen grains (Goldberg et al., 1993; Beals and Goldberg, 1997). Failure of anther rupture can lead to male sterility.

Cytoplasmic Male Sterility. Cytoplasmic male sterility (CMS) is a maternally inherited and male-expressed phenotype in which the plant fails to produce viable pollen grains and is thus an important agricultural trait (Levings, 1993; Schnabe and Wise, 1998). In a maize CMS line, cms-T, mitochondrial DNA rearrangement created a novel gene, *T-urf13*, which encodes an inner mitochondrial membrane protein that induces ion leakage and dissipation of membrane potential (Dewey et al., 1986, 1987, 1988; Holden and Sze, 1987). In cms-T plants, the tapetum undergoes precocious vacuolation and degeneration, and pollen development is blocked significantly earlier than in normal plants. In these cms plants, the major defect is on male sterility, and this has been attributed to the extraordinary metabolic demand on mitochondrial functions in anther cells during development (Levings, 1993). In sunflower PET-CMS, through DNA rearrangement a novel gene (orf522) has been fused to the 3′ end of the mitochondrial atpA gene. The two genes are expressed as a dicistronic transcript (Gagliardi and Leaver, 1999). In PET-CMS tapetal cells, DNA fragmentation occurs in concert with the condensation of young microspores. Cytochrome c release from intact mitochondria into the cytosol can be detected before these visible cell death symptoms and mitochondrial decline (Balk and Leaver, 2001), suggesting the involvement of the CMS mitochondria in the premature tapetal cell death. Interestingly, although RNA degradation is usually associated with cell death, degradation of the orf522 actually restored fertility, suggesting a deleterious role for the orf522 gene product (Gagliardi and Leaver, 1999).

Pollination and Fertilization. Pollination and fertilization are also associated with cell deterioration and death in various female tissues (Wu and Cheung, 2000). Pollen tubes, the structure derived from pollen grains to deliver the male germ cells to the embryo sac, penetrate several female tissues on their way to the egg cell. Prior to pollination, cells in the mature stigmatic papillae and the underlying glandular zone, the first layers of female tissue penetration by the pollen tubes, degenerate and release large amounts of cellular materials to the extracellular space. This facilitates pollen

grain germination and tube penetration of pistil tissues. Pollen tube growth deeper into the female tissues is accompanied by severe cell degeneration and death in what is known as the transmitting tissue (Wang et al., 1996), which provides nutrient resources and guidance cues for the passing pollen tubes (Cheung et al., 1995; Wu et al., 1995). This releases large amounts of nutrient resources as well as hydrolytic enzymes, both beneficial for the penetrating pollen tubes. The degeneration of the female tissues has also been postulated to be a defense mechanism to ablate a passage that could be used by pathogens to invade the ovary (Herrero, 1992). This pollination-induced female cell death is highly specific since adjoining cortical cells remain intact. Regulated RNA degradation has been suggested as one of the underlying mechanisms for the observed cell deterioration process (Wang et al., 1996).

Development of the female gametophyte also involves death in selected haploid precursor cells, leaving only one to undergo three rounds of mitosis, nuclear migrations and fusion, and cellularization to produce the typical seven-cell, eight-nucleate embryo sac (Russell, 1979, 1993). Degenerating haploid cells show shrinkage, cytoplasmic disorganization, and pycnotic nuclei (Bell, 1996). As the embryo sac develops, surrounding tissues also degenerate.

Programmed cell death in early female gametophyte development ensures the production of an egg cell that is protected deep inside the ovule within the embryo sac. To ensure that sperm cells are provided with access and guidance to the embryo sac for fertilization, an equally important cell death process is also programmed within the embryo sac (Russell, 1996). A pair of cytologically and probably functionally equivalent cells called the synergid cells occupy the entrance to the embryo sac. Death in one, or sometimes both, of these cells either precedes or accompanies the entrance of the pollen tubes and is believed to provide guidance cues for their entrance into the female chamber (Higashiyama et al., 2001; Cheung and Wu, 2001). In the degenerating synergid cell, the cytoplasm condenses, the nucleus becomes distorted, the vacuole collapses, and organelle degeneration, autolysis of cellular content, and cell membrane disintegration follow. $Ca^{2+}$ emitted from the ruptured synergid cell is speculated to be connected with pollen tube entrance.

In compatible pollination, fertilization occurs after pollen tube ruptures inside the embryo sac to deliver the male germ cells. But death in pollen tubes occurs significantly earlier in self-incompatible pollination in which the development of a pollen tube is arrested by specific female factors to prevent self-fertilization (see McCubbin and Kao, 2000). Pollen tubes elongating in incompatible pistil tissues develop thick cell walls, their tip swells, cellular inclusions lose their integrity, some tubes burst, and others are enveloped by cell wall materials at their tips. RNases are key factors in the arrest of self-incompatible pollen tubes in many systems (see McCubbin and

Kao, 2000). In poppies, DNA fragmentation as detected by TUNEL assays occurs in self-incompatible pollen tubes. Treatment with a caspase inhibitor appears to reduce the level of DNA fragmentation in these tubes (Jordan et al., 2000).

## Seed Development and Germination

Endosperm development in cereal seeds depends on cell death events that lead to the dormancy of endosperm cells in the outermost aleurone layer and the death of the more internally located but starchy endosperm. The aleurone cells will be activated by germination. Hydrolytic enzymes are secreted by the aleurone to break down reserves stored in the dead starchy endosperm cells to support seedling growth (Young and Gallie, 2000). The utilization of resources in the endosperm by aleurone cells is also accompanied by cellular and biochemical events marking progressive cell death (Fath et al., 2000). The cell death events in maize and wheat endosperm development begin when the biosynthetic phase of endosperm development is close to a conclusion and the pattern of cell death progression has been described in detail on the histological level (Kowles and Phillips, 1988; DeMason, 1997). On the molecular level, it is accompanied by DNA fragmentation. DNA degradation apparently is already initiated during the biosynthetic phase of endosperm development. Very large DNA fragments (above 50 kbp and up to several hundred kbp) are detectable before visible signs of cell death can be observed. The appearance of internucleosomal fragmentation accompanies late stages of cell death (Young and Gallie, 1999). Cell death in the starchy endosperm proceeds in close spatial proximity with the developing embryo and the aleurone layer, which remain alive. The level and activity of nucleases increase during endosperm development throughout the developing seeds. But the level of nucleases involved in endosperm cell death is between 5- to 10-fold higher than in the embryo (Young and Gallie, 2000). Clearly, the tight regulation of compartmentalized events exists in different tissue types differentiating within a developing seed. Cell death in the starchy endosperm is believed to facilitate access of hydrolases released from the aleurone for rapid mobilization of resources during germination.

Upon germination, the aleurone cells are activated to secrete hydrolytic enzymes to mobilize resources from the starchy endosperm for early development. As opposed to the starchy endosperm cells, aleurone cells undergo PCD only upon germination or activation by gibberellic acid under tissue culture conditions (Fath et al., 2000), and this process can be delayed by abscisic acid. The cell death process involves vacuolation, loss in cell membrane integrity, and ultimately cellular autolysis. Although cell death is accompanied by the accumulation of a large variety of nucleases and nucleases, the degradation of nuclear DNA into internucleosomal size fragments

is said not to occur, a phenomenon clearly distinct from the death of the starchy endosperm cells (Young and Gallie, 2000).

## PCD in Response to Environmental Stress

Some examples of PCD induced in response to extreme physical conditions, that is, abiotic stress, exist in plants. The adaptation of plants to environmental conditions such as high light and low humidity often involves covering their surfaces with a thick layer of dead hair cells. These are thought to undergo PCD, resulting in the formation of a protective layer that functions to block damaging sunlight and trap humidity (Greenberg, 1996). Submerged roots, stems, and petioles often suffer from a condition of low oxygen tension. In order to facilitate the transfer of gas through the root, patches of root cells undergo PCD to form pockets and channels of air. This tissue is called aerenchyma. Recently, the plant hormone ethylene was implicated in regulating this cell death process. Aerenchyma may also be formed by differential cell growth. This process may accompany the normal development of some plant species and may not be the result of anoxic conditions (Lam et al., 2000).

UV, ionizing radiation, and oxidative stress are also thought to induces PCD in plants (Lam et al., 2001). Oxidative stress induced by different compounds such as paraquat, a herbicide that enhances the rate of superoxide generation in cells, or by ionizing radiation, induces PCD in plants. Recently, it was reported that the animal antiapoptotic genes Bcl-$X_L$ and CED-9 can inhibit paraquat- and radiation-induced cell death in plants (Mitsuhara et al., 1999). Other environmental stresses such as high light, salt stress, and certain toxins were shown to induce cell death that was accompanied by DNA laddering, suggesting that they may involve some processes similar to apoptosis in animals (Mittler, 1998).

## Pathogen-Induced PCD

Pathogens are microorganisms capable of penetrating the plant's preexisting structural and chemical barriers and multiplying within plant tissues (Goodman and Novacky, 1994). The interaction of plants with pathogens can generally result in one of two outcomes: (1) The pathogen will infect the plant, multiply, and cause a disease. (2) The plant will rapidly mount a defense response that will block the spread of the pathogen, and prevent the disease (Nurnberger and Scheel, 2001). The outcome of this interaction is dependent on a set of plant-encoded genes (resistance genes) and a set of pathogen-encoded genes (avirulence genes), and is termed gene-for-gene (Bent, 1996). Gene-for-gene interactions determine whether the plant will

recognize the pathogen and activate its defenses, thereby become resistant, or will fail to recognize the pathogen, will not activate defense mechanisms, and become infected (Hammond-Kosack and Jones, 1996). Interestingly, some of the plant's gene-for-gene genes were found to be similar to animal genes controlling bacterial pathogenicity and PCD (Rahme et al., 1997; Inohara and Nunez, 2001).

Different types of PCD are activated during plant–pathogen interactions. During the development of disease, symptoms such as cell death can appear. For years it was thought that these are the result of toxins produced by pathogens during the infection process (Goodman and Novacky, 1994). However, in recent years it became apparent that this type of cell death actually results from the activation of a PCD pathway. Thus, mutants that activate symptom-associated cell death in the absence of a pathogen were isolated (Greenberg, unpublished). In addition, the expression of antiapoptotic genes was found to inhibit certain symptom-associated cell deaths (Dickman et al., 2001), and some toxins produced by pathogens were shown to induce PCD when applied to plant cells in the absence of the pathogen (Wang et al., 1996).

During plant–pathogen interactions that result in the successful inhibition of pathogen growth, a different type of PCD is activated. This type of PCD is activated in infected cells or in cells that surround the infection site and result in the formation of a lesion, called the hypersensitive response (HR) lesion (Dangl et al., 1996). During the HR cells undergo a very rapid process of cell death (hence, the name hypersensitive) and prevent the pathogen, mostly an obligate parasite such as a virus or certain types of bacteria and fungi, from replicating, because these require living cells for their proliferation. Due to the association of this type of cell death with disease resistance, it has been the focus of considerable research (in contrast to symptom-associated cell death), and will be described in more detail below.

PCD activated during the HR can be very successful in preventing obligate parasites from replicating within living cells. However, it was found that some pathogens which feed on dead plant tissue (necrotrophic pathogens) deliberately cause the activation of this PCD pathway in plants in order to propagate on the resulting dead plant tissue (Govrin and Levine, 2000).

## PCD Induced during the Hypersensitive Response

The recognition of an invading pathogen by the plant, that is, the recognition of an avirulence *Avr* factor by the resistance *R* gene, triggers a signal transduction pathway that results in the induction of multiple defense pathways, including PCD (Fig. 2; Godiard et al., 1994). Some of the early events associated with the induction of this response are the rapid generation of reactive oxygen species (ROS) in the form of superoxide and $H_2O_2$, the so-

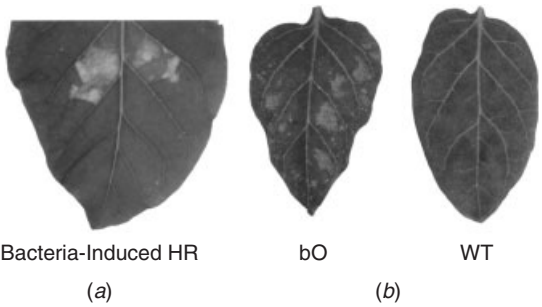


FIGURE 2. Pathogen-induced PCD. (A) Induction of PCD in tobacco by a bacterial pathogen (after Mittler et al., 1999). (B) Induction of PCD in tomato by expression of a bacterial proton pump (bacterio-opsin; bO; after Mittler and Rizhsky, 2000).

called oxidative burst (Jabs et al., 1996; Tenhaken et al., 1995; Grant and Loake, 2000), and a rapid flux of ions across the plasma membrane (Mittler et al., 1995; Jabs et al., 1997). The rapid generation of ROS by plant cells upon the recognition of pathogens may be analogous to the production of ROS by macrophages. Moreover, it is thought that a membrane-bound NADPH oxidase complex is involved in this process (Hammond-Kosack and Jones, 1996). Thus, much like macrophages that produce ROS to kill pathogens, plant cells may also generate ROS at a very high level. These may function to kill the pathogen, the plant cell, or both. Initially, it was thought that ROS are sufficient to induce PCD. However, it was recently shown that nitric oxide (NO) is required for the activation of PCD by ROS (Delledone et al., 2001; Klessig et al., 2000). In addition to inducing PCD, ROS such as $H_2O_2$ function as signals that activate other defense mechanisms, that is, pathogenesis-related (PR) proteins (Linthorst, 1991), and phytoalexins, and as oxidizing agents that cause the cross-linking and strengthening of cell walls (Hammond-Kosack and Jones, 1996). It should however be noted that the oxidative burst alone may not be sufficient to trigger cell death in all plant–pathogen systems (Glazener et al., 1996; Delladone et al., 2001).

The enhanced production of ROS during the HR is accompanied by the suppression of antioxidative enzymes such as ascorbate peroxidase and catalase. This suppression occurs at the activity level by salicylic acid (SA) and NO (Klessig et al., 2000), as well as at the gene expression level (Mittler et al., 1999). The process of ROS production during the HR is therefore enhanced due to the suppression of the cellular antioxidative mechanism. Thus, ROS produced by NADPH oxidases and other cellular sources during the HR can accumulate to very high levels because their removal is suppressed.

Under conditions of low oxygen pressure or high humidity, cell death may be inhibited without affecting the activation of other defense mecha-

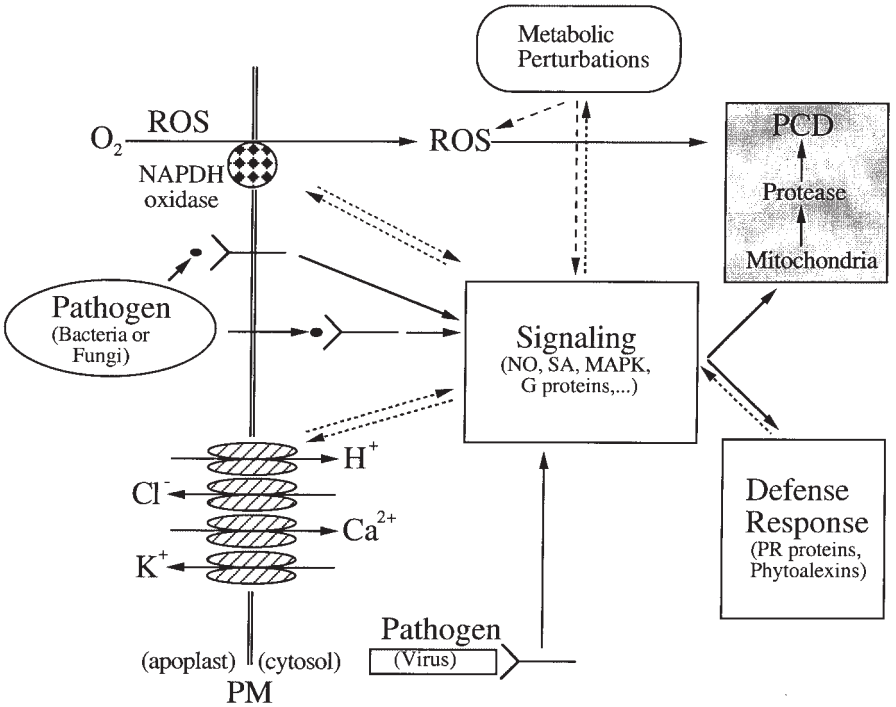


FIGURE 3. A model depicting some of the signaling pathways activated in plants in response to pathogen attack. Different pathogens are shown to be recognized by plant receptors. These activate a signal transduction pathway that results in the inward flux of $Ca^{2+}$ and protons, the enhanced generation of ROS, and the activation of a MAPK cascade(s). The plant hormones SA and NO, and ROS coordinate the induction of PCD and defense mechanism in infected cells and in neighboring cells.

nisms such as the synthesis of SA and the induction of PR proteins (Mittler et al., 1996; Hammond-Kosack and Jones, 1996). These results suggest that PCD can be uncoupled from the activation of other defense mechanisms during the HR. This hypothesis is supported by previous studies in which the activation of defense mechanisms was shown to occur in the absence of PCD (Hammond-Kosack et al., 1996). Therefore, although the signal transduction events that lead to the activation of PCD and defense mechanisms during the HR are controlled by a single gene-for-gene interaction, it appears as if two independent pathways may be set into motion by this recognition event, a pathway for PCD and a pathway for the induction of PR proteins and perhaps other defense mechanisms (Fig. 3).

The signal transduction pathway that leads to the activation of PCD was shown to involve an increase in proton and $Ca^{2+}$ flux into the cytosol, and the activation of a MAPK cascade (Jabs et al., 1997; Zhang and Klessig, 2001). The

increased intracellular concentration of $Ca^{2+}$, accompanied by the acidification of the cytoplasm (inward increase in proton flux), is thought to signal the activation of different kinases and the phosphorylation of several cellular targets. These may include the phosphorylation and activation of the NADPH oxidase complex (Dangl et al., 1996). Several studies using mutants altered in the activation or propagation of PCD in plants (lesion mimic mutants; Walbot et al., 1983; Dangl et al., 1996; Loake, 2001), suggest that plants contain a set of genes encoding proteins that suppress PCD at different levels, and a set of proteins that induce PCD, depending on the type of environmental signal perceived. Unfortunately, although some of these genes were cloned (Lam et al., 2000), little is known about how they function.

Lesion mimic mutants are mutants that spontaneously activate the HR in the absence of a pathogen (Dangl et al., 1996). They are often referred to as "accelerated cell death" (*acd*; Greenberg and Ausubel, 1993), or "lesion simulating disease" (*lsd*; Dietrich et al., 1994) mutants. The mutations that cause the appearance of HR lesions in the absence of a pathogen in these plants are thought to occur in plant genes that control PCD. Thus, mutations in these genes would result in the abnormal activation or suppression of pathogen-induced PCD (Greenberg and Ausubel, 1993; Dietrich et al., 1994; Greenberg et al., 1994). Disease lesion mimic mutants have been isolated from tomato, maize, barley, rice, and *Arabidopsis*. They were classified according to their appearance into two groups: initiation, and feedback or propagation mutants (Walbot et al., 1983; Dietrich et al., 1994). Several cell death mutants express molecular and biochemical markers associated with the antimicrobial defense response of plants. These include PR proteins, the accumulation of SA, the deposition of callose or other cell wall strengthening compounds, and the synthesis of phytoalexins (Dietrich et al., 1994; Greenberg et al., 1994). The activation of these antimicrobial defenses in the absence of a pathogen further indicates that the cell death pathway activated in these mutants is the same pathway activated during the response of plants to invading pathogens.

Cell death mutants are powerful tools for the study of PCD in plants. By crossing these mutants for complementation studies, the order of the cell death genes along the PCD pathway may be determined (Dangl et al., 1996; Rusterucci et al., 2001; Loake, 2001). For example, it was recently reported that LSD1, a novel zinc finger protein that regulates PCD induced by superoxide or pathogen infection, downregulates a PCD pathway that is driven by ROS, SA and the plant genes PAD4, EDS1, and NPR1. This pathway is activated in cells that surround the initial infection site. Thus, when LSD1 is mutated, it can no longer suppress PCD in these cells and the lesion that is formed upon infection or ROS application becomes a runaway cell death process that spreads to the entire leaf instead of being confined to only a few cells (Fig. 4; Rusterucci et al., 2001; Loake, 2001).

Additional tools that may prove beneficial for the study of PCD in plants are transgenic plants that spontaneously activate the HR in the absence of a

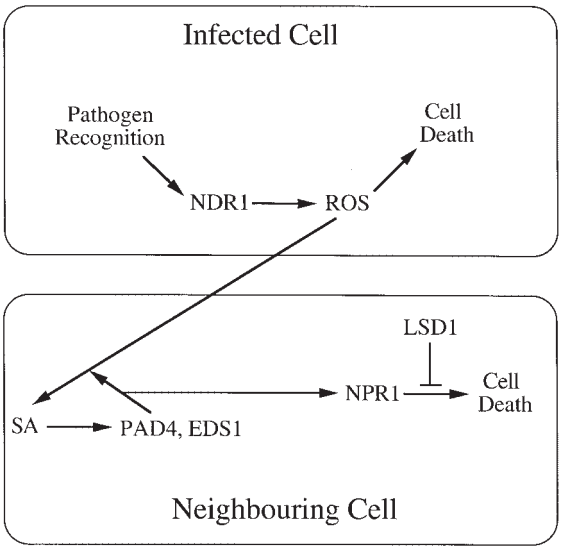


FIGURE 4. A model showing the PCD-inhibiting function of LSD1. LSD1 is shown to suppress PCD in cells that surround the primary infected cells, and therefore to limit the spread of cell death and create a lesion with a defined border. In the absence of this gene, PCD is not suppressed and a runaway PCD process is initiated. In *lsd1* plants this process will continue until the entire leaf is dead. See also Loake (2001).

pathogen. For example, transgenic tobacco plants that express a bacterial gene encoding the proton pump bacterio-opsin (bO) were found to spontaneously activate the HR and defense mechanisms in a manner similar to some of the dominant initiation cell death mutants (Fig. 2; Mittler et al., 1995). The expression of other transgenes was also reported to cause the activation of the HR (Mittler and Rizhsky, 2000). The expression of some of these genes is thought to drastically alter the metabolism of the cell. These alterations may result in the activation of PCD similar to the activation of PCD by alterations in cellular homeostasis in animals (Dangl et al., 1996; Mittler and Rizhsky, 2000). In support of this hypothesis, the majority of disease lesion mimic mutants appear to result from dominant mutations. It was therefore suggested that some of these mutations alter the homeostasis of the cell, thus, generating a cell death signal (Walbot et al., 1983).

## Molecular Machinery of PCD in Plants

The sequencing of the entire genome of the flowering plant *Arabidopsis thaliana* failed to identify plant homologs of key regulators of PCD in animals such as caspases and Bcl-2 or Bax. Plants were however found to contain a

group of proteases called metacaspases, and homologs of BI-1 (Bax inhibitor-1) and DAD-1 (Lam et al., 2001). There are two types of metacaspases in plants: Type I that contains a predicted caspaselike proteolytic domain but lacks the death effector domain, and Type II that contains in addition to the caspaselike domain an N-terminal zinc finger and proline-rich domains, also found in LSD-1, a protein involved in the control of PCD during the HR (see above). Molecular and pharmacological studies support the involvement of caspases in PCD in plants (possibly mediated by the metacaspase family). These include the suppression of HR-associated PCD by synthetic peptides that act as inhibitors of caspase activity, and measurements of caspaselike protease activity in plant cells undergoing HR-PCD (Lam et al., 2001). Thus, in plants metacaspases may play a role similar to that of caspases in animals. Additional players that may be similar to some of those controlling PCD in animals are small GTP-binding proteins of the Ras class and cysteine-sensitive proteases (Ono et al., 2001; Lam et al., 2000, 2001).

Although Bax homologs were not found in plant cells, the expression of Bax in plants induces PCD (Kawai-Yamada et al., 2001). Moreover, this induction requires the proper oligomerization and cellular localization of Bax in plants. The animal protein BI-1 was recently found to inhibit Bax toxicity in yeast. Homologs of this protein were found in plants. Coexpression of Bax and the plant homolog of BI-1 resulted in the inhibition of Bax-induced cell death in plants (Kawai-Yamada et al., 2001). These results, as well as the finding that mitochondrial permeability transition (MPT) and release of cytochrome c from mitochondria accompany certain types of PCD in plants (Balk and Leaver, 2001; Curtis and Wolpert, 2002), suggest an active role for the mitochondria in plant PCD processes. Further support for this hypothesis comes from overexpression of animal antiapoptotic genes such as Bcl-2, Bcl-$X_L$, and CED-9 in plants. These were found to inhibit pathogen-induced PCD and oxidative stress-induced PCD in plants (Mitsuhara et al., 1999; Dickman et al., 2001). A recent homology search using BI-1 sequences revealed the possible existence of a functional homolog of Bcl-2 in plants (called ABR proteins; Lam et al., 2001). However, further experimental work is required to support this possibility. A model illustrating some of the events associated with plant PCD is shown in Fig. 5.

Interestingly, when the different animal antiapoptotic genes were ectopically overexpressed in plants, they did not appear to affect certain developmental processes that require PCD such as xylem formation, suggesting that different PCD pathways in plants may be controlled by different mechanisms.

## Summary

PCD is a basic biological process essential for the survival of almost all living organisms. The many examples of PCD in plants may suggest that plants

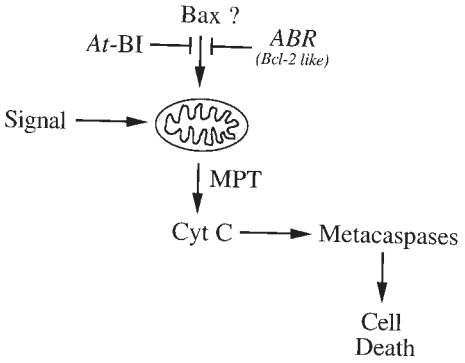


FIGURE 5. A model showing some of the players involved in regulating PCD in plants. Abbreviations: ABR, *At*-BI-2-related proteins; *At*-BI, *Arabidopsis thaliana* Bax inhibiotor; cyt-c, cytochrome c; MPT, mitochondrial permeability transition.

use different pathways for PCD. These may be activated by different developmental signals, environmental insults, hormones, toxins, or pathogens. PCD induced by different agents may require different modes of cell death. For example, the death of an infected plant cell should occur in a relatively rapid rate to prevent the spread of the invading pathogen, whereas the death of a senescing cell should occur at a relatively slow rate that will allow the efficient transfer of nutrients from the dying cell to younger cells. In addition, different cell deaths in plants are inhibited by different proteins. For example, pathogen-induced PCD can be inhibited by the expression of Bcl-2, whereas developmental PCD of xylem vessels is not. Although plants and animals use PCD for similar purposes, many differences exist between PCD in plants and PCD in animals. It is possible that these result from the different anatomical and physiological characteristics between plants and animals.

Most PCD strategies utilized during plant development are likely to involve variations in themes around a relatively conserved cellular, molecular, and biochemical framework. The stimulus that triggers each individual cell death program is expected to differ, although various phytohormones may overlap in their roles as stimulating or suppressing agents for individual processes (Quirino et al., 2000; Rubinstein, 2000; Young and Gallie, 2000; Fukuda, 2000; Fath et al., 2000). Their exact functional roles and how they initiate the cell death programs remain to be understood. The story of *early signaling pathways* of developmentally regulated PCD is almost an untouched territory. A heterotrimeric G protein has been implicated in the senescence of the orchid *Phalaenopsis* (Porat et al., 1994). Rac-like small GTPases have been shown to mediate pathogen-induced PCD (Ono et al., 2001) and in secondary cell wall differentiation in cotton, a terminal differ-

entiation process (Potikha et al., 1999). We have observed that tobacco and *Arabidopsis* Rac-like GTPases are mediators for auxin responses (Tao et al., 2002). This suggests the potential use of a large family of molecular switches in mediating endogenous as well as environmental signals to activate developmental PCD pathways.

Interestingly, at least some mechanisms of PCD appear to be conserved between yeast, plants, and animals. These appear to involve as a central player the mitochondrion. Because the mitochondrion is believed to have originated from an endosymbiotic event between a primitive eukaryotic cell and a proteobacteria, the finding of different PCD processes in bacteria (Lewis, 2000) may extend the link between yeast, plants, and animals, to include bacteria and mechanisms of PCD in bacteria that involve the rupture of bacterial cells. PCD appears therefore to be a key mechanism for the survival of almost all known organisms.

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CHAPTER

6

# REGULATION OF APOPTOSIS BY EXTRACELLULAR MATRIX DURING POSTEMBRYONIC DEVELOPMENT IN *XENOPUS LAEVIS*

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Programmed cell death or apoptosis is an integral part of animal development. It is essential for the establishment and maintenance of tissue structure and organ function and is precisely controlled both temporally and spatially during development (Jacobson et al., 1997; Meier et al., 2000). Some cells, such as the neurons, are produced in large excess early in development and the unwanted ones, that is, those fail to find target cells, are eliminated through apoptosis later. Another major type of physiological cell death is responsible for the removal of the organs/tissues that serve specific roles only during certain developmental periods but are not needed in adult life. In addition, apoptosis is also important for the high levels of plasticity in the development of many animal species by compensating for genetic and/or environmental changes.

Anurans are among the earliest models used for developmental studies in vertebrates where apoptosis is obviously critical for the formation of the adults (Dodd and Dodd, 1976; Gudernatch, 1912; Shi, 1999). They undergo biphasic development, embryogenesis to generate a free living tadpoles, and subsequent metamorphosis to produce the adults with a new habitat (Dodd

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and Dodd, 1976; Shi, 1999). The metamorphic process changes essentially every single tissue/organ. Some develop de novo, whereas others resorb completely. The majority of the tissues/organs undergo partial but often drastic transformations to adapt to their new roles in the adult. All these diverse changes are, however, under the control of thyroid hormone (TH) (Dodd and Dodd, 1976; Shi, 1999). The addition of TH to the rearing water of premetamorphic tadpoles can induce precocious changes, while blocking the synthesis of endogenous TH leads to the formation of giant tadpoles that fail to undergo the transformation. Furthermore, most, if not all, of these changes are organ autonomous as in vitro cultures of tadpole organs, such as the limb and intestine, can metamorphose in response to TH added to the culture medium (Ishizuya-Oka and Shimozawa, 1991; Tata et al., 1991). Such properties have made anuran metamorphosis an attractive model to study postembryonic development in vertebrates. Here we review the involvement and regulation of apoptosis during *Xenopus laevis* metamorphosis. We focus primarily on the remodeling of the animal intestine and the role of the extracellular matrix (ECM) in this process.

## Apoptosis during Metamorphosis

The organ autonomous nature of anuran metamorphosis indicates that the TH-dependent process is genetically predetermined in different organs. Furthermore, as early as 1966, Tata (Tata, 1966) showed that both protein and RNA synthesis are required for tail resorption, indicating that tail resorption involves programmed cell death. Direct demonstration that apoptosis occurs during metamorphosis came when Kerr and coworker (Kerr et al., 1974) examined electron microscopically the resorption of the tail muscle and epidermal cells during metamorphosis of the dwarf tree frog *Litoria glauerti*. Their studies indicated that these two major cell types of the tail undergo a series of well-defined, sequential morphological changes of apoptosis, including the condensation of the cytoplasm, segregation of the compacted chromatin into dense masses that lie against the nuclear envelope, and the subsequent formation of apoptotic bodies. Similar findings have also been reported for *Rana japonica* (Kinoshita et al., 1985) and *Xenopus laevis* (Nishikawa and Hayashi, 1995) (Fig. 1).

Cell death is also important for the de novo development of the limbs. As the limbs undergo morphogenesis, the interdigital cells are selectively removed, although it remains to be shown that they undergo apoptosis (Fig. 1).

Intestinal remodeling represents the third type of transformation during metamorphosis. Here, an existing organ is converted into an adult organ with a drastically reduced size but more complex cross-sectional morphology to accommodate the dietary change from being herbivorous to being

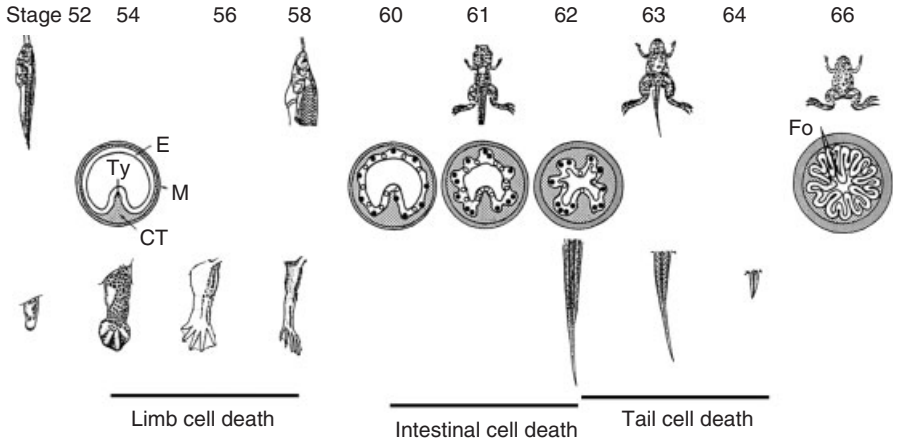


FIGURE 1. Stage-dependent apoptosis in different organs during *Xenopus laevis* metamorphosis. The developmental stages are from Nieuwkoop and Faber (1956). The tails at stages 62 through 66 are drawn to the same scale to show the resorption (no tail remains by stage 66), whereas the tadpoles, intestinal cross sections (middle), and hindlimbs at different stages are not in the same scale in order to show the morphological differences. Tadpole small intestine has a single epithelial fold (the typhlosole, Ty), where connective tissue (CT) is abundant, while a frog has a multiply folded intestinal epithelium (E), with elaborate connective tissue and muscle (M). Fo: epithelial folds. Dots: proliferating adult intestinal epithelial cells. Open circles: apoptotic primary intestinal epithelial cells.

carnivorous. The tadpole intestine is a simple tubular structure consisting of predominantly the larval epithelial cells surrounded by sparse connective tissue and muscles (Shi and Ishizuya-Oka, 1996). During metamorphosis, the larval epithelium undergoes complete degeneration and is replaced by adult epithelium (Fig. 1). Morphological and biochemical analyses indicate that the larval epithelial cells undergo apoptosis and the apoptotic bodies are removed at least in part through phagocytosis by macrophages migrating over from the connective tissue (Ishizuya-Oka and Shimozawa, 1992b; Ishizuya-Oka and Ueda, 1996). Accompanying this apoptosis is the proliferation of the adult epithelial cells, whose origin remains unclear, followed by their differentiation and morphogenesis to form the multiply folded adult epithelium. Concurrently, elaborate connective tissue and muscles develop around the adult epithelium (Fig. 1).

In vitro studies suggest that TH can induce cell death by targeting the dying cells directly at least for some larval cells when cultured in vitro. This was first shown by Yoshizato and colleagues for tail epidermal cells (Nishikawa et al., 1989; Nishikawa and Yoshizato, 1986). They isolated the epidermal cells from the tail of *Rana catesbeiana* tadpoles and cultured them

in vitro in the presence or absence of TH. Although these cells survived well and proliferated in the absence of TH, the addition of TH to the culture medium caused these cells to die, suggesting that the TH-dependent epidermal cell death is cell autonomous, at least when cultured in isolation in vitro.

Similarly, we isolated the larval epithelial cells and the fibroblasts from the intestine of *Xenopus laevis* tadpoles and cultured them in vitro (Su et al., 1997a, 1997b). Our results showed that the larval epithelial cells were induced to die by physiological concentrations of TH. On the other hand, the fibroblasts were refractory to TH-induced death, although both the fibroblasts and epithelial cells were capable of proliferation in vitro and TH stimulated their DNA synthesis. Biochemical and morphological analyses indicated that the TH-induced epithelial cell death had all the characteristics of apoptosis, including the formation of apoptotic bodies and nucleosomal sized nuclear DNA fragments. Although these studies suggest a cell autonomous response to TH for isolated larval cells, in vivo, apoptosis likely involves both direct action of TH in the dying cells as well as indirect effects through cell–cell and cell–ECM interactions as described below.

## ECM Remodeling and Cell Fate Determination

The vast majority of the cells in an organism are in constant contact with the ECM. The ECM not only provides the essential physical support for cells that constitute individual organs but also serves as a media for cell–cell interactions/signaling (Hay, 1991). In addition, the ECM can signal cells directly through many cell surface receptors that bind to ECM components (Brown and Yamada, 1995; Schmidt et al., 1993). Finally, numerous extracellular factors such as growth factors and morphogens are stored in the ECM and their availability to signal nearby cells depends on the nature of the ECM. Thus, changes in the ECM can result in two types of signals for the nearby cells: direct signaling through cell surface ECM receptors and indirect effects through alterations in cell–cell and cell–extracellular factor interactions.

The drastic changes in various organs during anuran metamorphosis undoubtedly involve extensive remodeling of the ECM. As early as 1962, Gross and colleagues (Gross and Lapiere, 1962) identified collagenase activity in the resorbing tail. Such activity is believed to be mainly responsible for the degradation of the collagen, a major component of the ECM in the tail, during tail resorption. However, little is known about whether and how ECM remodeling may play a role in various metamorphic events.

We have been studying the remodeling of the intestine during *Xenopus laevis* metamorphosis. This process involves the complete degeneration of the larval epithelium and de novo development of the adult epithelium. Concurrently, the underlying connective tissue and outer muscle layers

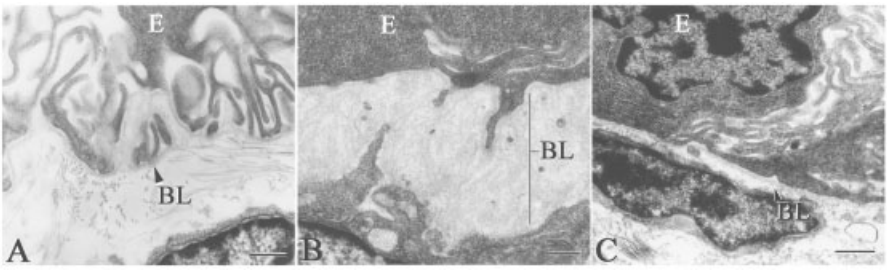


FIGURE 2. Electron micrographs showing the remodeling of the basal lamina (BL) just beneath the intestinal epithelium (E) during metamorphosis. (A) at stage 56; (B) stage 61; (C) stage 66. Bars: 0.5μm.

develop extensively. In between the epithelium and the connective tissue is a distinct ECM, the basal lamina, which is composed of laminin, entactin, collagens, and proteoglycans, and so on (Hay, 1991; Timpl and Brown, 1996). As the epithelium undergoes metamorphic transformations, one would expect that the basal lamina also undergoes remodeling. Indeed, electron microscopic examination shows that in premetamorphic *Xenopus laevis* tadpoles, the basal lamina is a thin but continuous structure (Fig. 2A). During metamorphosis, it becomes thick (Fig. 2B) and remains so until the larval epithelium finally disappears through massive apoptosis (Ishizuya-Oka and Shimozawa, 1992b; Shi and Ishizuya-Oka, 1996). Interestingly, the basal lamina appears to be much more permeable at the climax of metamorphosis (stages 60–63) in spite of the increased thickness. This permeability is reflected by the frequently observed migration of macrophages across the ECM into the degenerating epithelium, where they participate in the removal of apoptotic epithelial cells. In addition, extensive contacts are present between the proliferating adult epithelial cells and the fibroblasts on the other side of the basal lamina. Larval epithelial cells are completely replaced by the adult epithelial cells around stages 62 and 63. After stage 63, with the progress of intestinal morphogenesis, that is, intestinal fold formation, the adult epithelial cells differentiate into absorptive epithelium. Concurrently, the basal lamina underlining the differentiated adult epithelium becomes thin and flat again (Fig. 2C). Such changes support the role of ECM in intestinal transformation.

Direct evidence for the role of ECM in influencing cell fate has come from in vitro studies. As described above, tadpole intestinal epithelial cells can be cultured in vitro and induced to undergo apoptosis by TH just as in vivo (Su et al., 1997a, 1997b). When the plastic culture dishes are coated with various ECM proteins, such as fibronectin and laminin, the cells become more resistant to TH-induced cell death. Consistent with these apoptosis-inhibiting effects of the ECM coatings, when proliferating/differentiating adult epithe-

lial cells of the intestine at stage 64 are cultured in vitro on plastic dishes, they also undergo TH-induced apoptosis. In vivo, these adult cells proliferate and differentiate instead of undergoing apoptosis in the presence of high levels of circulating plasma TH. Thus, dissociating the adult cells from the ECM alters their response to TH. On the other hand, the ECM components do not influence TH-stimulated cellular DNA synthesis and the downregulation of two epithelial specific genes in the primary culture of the intestinal epithelial cells. Thus, the ECM preferentially affects cell death.

A role for cell–cell interactions in metamorphosis is also supported by organ culture studies. For example, when tail fragments are cultured in vitro, TH can induce cell death and tail resorption. The removal of tail epidermis prevents TH-induced tail resorption in organ cultures (Niki et al., 1982; Niki and Yoshizato, 1986). On the other hand, there is also evidence to suggest that adult-type non-T leukocytes may participate in the specific elimination of larval tail cells (Izutsu and Yoshizato, 1993; Izutsu et al., 1996). In addition, when intestinal fragments are cultured in the presence of TH, larval cell death takes place within 3 days, followed by the development of adult epithelium after 5 days (Ishizuya-Oka and Shimozawa, 1991). When isolated epithelium is cultured with TH, only cell death occurs. The development of the adult epithelium requires the presence of the connective tissue as cocultures of the larval epithelium and the connective tissue restore adult epithelial development (Ishizuya-Oka and Shimozawa, 1992a). Thus, cell–cell interactions, which are dependent on and/or influenced by the nature of the ECM, are likely important for both larval tissue degeneration and adult tissue development.

## Gene Regulation during Metamorphosis

The biological effects of TH are believed to be mainly manifested through the regulation of direct TH response genes by thyroid hormone receptors (TRs). TRs belong to the superfamily of nuclear receptors that includes steroid hormone receptors and 9-*cis* retinoic receptors (RXRs), and the like (Mangelsdorf et al., 1995; Sachs and Shi, 2000; Shi and Ishizuya-Oka, 2001; Tsai and O'Malley, 1994; Yen, 2001). They are transcription factors that recognize specific DNA sequences, or TH response elements (TREs), in their target genes. Both TR monomers and dimers can bind to TREs. In vitro and in vivo studies show that TRs most likely function as heterodimers formed with RXRs in vivo. In the absence of TH, TR/RXR heterodimer represses the expression of TH-inducible genes. Upon binding by TH, TR/RXR switches from being a repressor to being an activator to upregulate the expression of the TH-inducible genes. Thus, the key to understanding how TH regulates anuran metamorphosis lies in identifying and functionally characterizing the TH-response genes within individual metamorphosing organs/tissues.

## TH Response Genes

To affect tissue transformations during metamorphosis, TH is presumed to induce a cascade of gene regulation. The earliest genes, or so-called direct response genes, are those regulated by TRs at the transcriptional level immediately after TH becomes available. The products of the genes, in turn, are likely to participate in the regulation of downstream genes either directly or indirectly. For the purpose of the discussion here, we refer to those genes whose mRNA levels are altered by a 24-hour TH treatment of premetamorphic tadpoles as early TH response genes, with the rest designated as late TH response genes.

Systematic isolation of early TH response genes has been carried out by using PCR-based subtractive differential screen for the hindlimb, tail, brain, and intestine of *Xenopus laevis* (Buckbinder and Brown, 1992; Denver et al., 1997; Shi, 1999; Shi and Brown, 1993; Wang and Brown, 1993). This had led to the identification and characterization of many genes in those organs. In general, there are many more genes upregulated by TH than those downregulated by TH. Of the four organs analyzed, the tail and intestine share more similarities with each other than with the other two. Both organs involve the predominantly apoptotic degeneration of larval tissues at early stages of metamorphosis. Many of the TH response genes are regulated by TH similarly in the two organs. Sequence analyses reveal that the genes belong to several different groups. The first groups include gene-encoding transcription factors, such as TRβ and NF-I. These transcription factors are expected to regulate the expression of the downstream genes directly, thus propagating the TH signal. The second groups contain genes that can affect cell–cell and cell–ECM interactions, for example, those encoding matrix metalloproteinases (MMPs), which digest various proteinaceous components of the ECM (see below). The rest encode various other enzymes or cell-specific proteins, and their functions during metamorphosis are likely to vary depending on the cells in which they are expressed.

Notably absent among the isolated early TH response genes are those encoding cell death effectors and regulators, such as caspases and Bcl-2 superfamily members (Adams and Cory, 1998; Cryns and Yuan, 1998; Rao and White, 1997; Shi et al., 1998). This may be because the mRNA levels of such genes do not change significantly during metamorphosis and thus escaped the PCR-based screens. Alternatively, the PCR-based screens are not saturated. Finally, the regulation of such genes by TH may be delayed relative to the early TH response genes. A PCR-based screen for genes regulated in the intestine after a 4-day TH treatment of premetamorphic *Xenopus laevis* tadpoles also failed to isolate any cell death genes (Amano and Yoshizato, 1998). Instead, it isolated many late TH response genes. These genes again fall into several categories, including transcription factors and signaling molecules, and their roles, if any, in apoptosis, remain to be investigated.

A caveat in this screen is, however, that after 4 days of TH treatment, apoptosis is largely completed in the intestine and the screen may, therefore, miss cell death genes.

## Expression of Cell Death Genes during Metamorphosis

The morphological and biochemical similarities between cell death during metamorphosis and that in other animal species suggest the existence of conserved molecular pathways. Consistently, TH-induced apoptotic DNA fragmentation in the primary cell cultures of the larval epithelial cells from *Xenopus laevis* tadpole intestine can be inhibited by known inhibitors of mammalian cell death, including inhibitors of caspases and nucleases (Su et al., 1997a, 1997b). Similarly, caspase inhibitors also blocked TH-induced apoptosis of a cell line derived from tadpole tail muscle (Yaoita and Nakajima, 1997).

The cloning of several members of the caspase family in *Xenopus laevis* has allowed the analysis of their expression profiles (Nakajima et al., 2000; Yaoita and Nakajima, 1997). Several caspases are expressed in the cell line derived form the tadpole tail muscles and most of them are upregulated by TH. Their upregulation, however, requires more than 1 day of TH treatment. Thus, they are most likely regulated indirectly by TRs. This is consistent with the fact that they are presumed to act late in the TH-induced apoptotic pathways and also explains why none of them were found in any differential screens for early response genes above.

All the cloned *Xenopus* caspases are expressed during tail resorption (Nakajima et al., 2000; Yaoita and Nakajima, 1997), although their mRNA levels and regulation during development vary. In general, they are upregulated during tail resorption and by prolonged TH treatment of premetamorphic tadpoles. Thus, they are presumably involved in the apoptotic degeneration of the tail. In the intestine, both caspase-1 and -3 are expressed at low levels prior to metamorphosis (before stage 58), but are upregulated moderately during intestinal remodeling (around stages 60–62) (Su, Amano, and Shi, unpublished data). Thus, the larval epithelial cell death may also involve caspases, consistent with the ability of a caspase inhibitor to block TH-induced DNA fragmentation in primary intestinal epithelial cell cultures (Su et al., 1997a, 1997b).

Two Bcl-2 family members have also been cloned in *Xenopus laevis* (Cruz-Reyes and Tata, 1995). They are highly homologous to those Bcl-2 family members capable of inhibiting apoptosis and are capable of doing so in tissue culture cells. Furthermore, transgenic expression of one of them, the *Xenopus* Bcl-xl, under the control of neural β-tubulin promoter indicates that it inhibits the death of some but not all neuronal cells during metamorphosis (Coen et al., 2001). These results suggest that Bcl-2 family members may

participate in cell death regulation during *Xenopus* development and that different mechanisms may be responsible for the degeneration of different larval cells. Unfortunately, little is known about the temporal and spatial profiles of the expression of these and other members of this superfamily. Thus, their possible roles during metamorphosis remain to be investigated.

## Roles of MMPs in TH-Induced Apoptotic Tissue Remodeling

ECM remodeling and degradation are mediated largely by matrix metalloproteinases (MMPs). MMPs are extracellular enzymes that are capable of degrading various components of the ECM (Alexander and Werb, 1991; Birkedal-Hansen et al., 1993; McCawley and Matrisian, 2001; Parks and Mecham, 1998). This growing family of enzymes includes collagenases, gelatinases, and stromelysins, and so on. They are secreted into the ECM as pro-enzymes with the exception of stromelysin-3 (ST3) and membrane-type MMPs, which appear to be secreted in the active form and membrane-bound, respectively (McCawley and Matrisian, 2001; Pei and Weiss, 1995). The pro-enzymes can be activated in the ECM through the proteolytic removal of their propeptide (Murphy et al., 1999; Nagase, 1998; Nagase et al., 1992). The mature MMPs are then capable of cleaving various components of the ECM with distinct but often overlapping substrate specificity. Thus, selective expression and/or activation of different MMPs will lead to distinct modifications of the ECM to influence cell behavior and tissue transformation.

### Differential Regulation of MMP Expression during Metamorphosis

The participation of MMPs in amphibian metamorphosis was first implicated over 30 years ago by the drastic increases in collagen degradation activity in the resorting tadpole tail (Gross and Lapiere, 1962). However, the cloning of frog MMP genes came only after two *Xenopus* MMP genes were identified as thyroid hormone-inducible genes from subtractive differential screens (Shi, 1999; Shi and Brown, 1993; Wang and Brown, 1993). The full-length proteins encoded by these two genes are highly homologous to their mammalian counterparts, collagenase-3 (Col3) and stromelysin-3 (ST3), respectively (Brown et al., 1996; Patterton et al., 1995), suggesting a functional conservation as well. Another frog MMP, the *Rana catesbeiana* collagenase-1, was cloned by screening an expression cDNA library with an antiserum against purified *Rana* tail collagenase (Oofusa et al., 1994), and this gene was shown to contain a TRE (Sawada et al., 2001). All three frog MMP genes as well as the subsequently cloned *Xenopus* collagenase-4 (Col4)

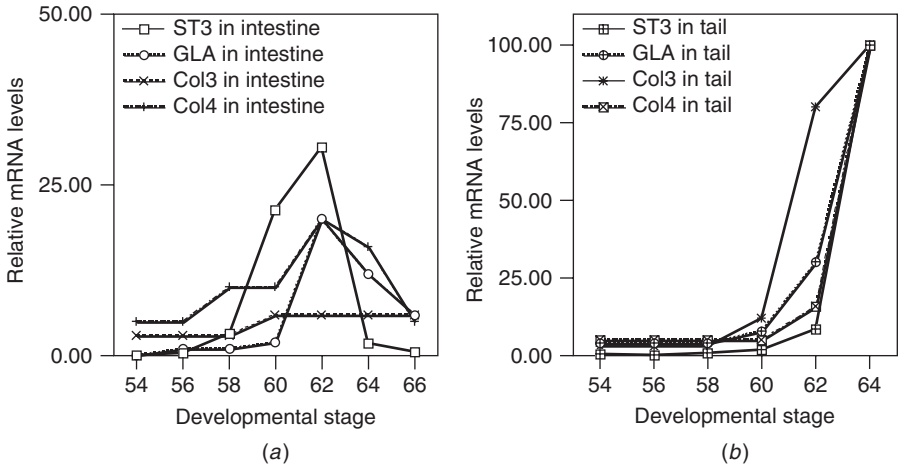


FIGURE 3. Differential regulation of MMP genes during intestinal remodeling (A) and tail resorption (B) in *Xenopus laevis*. The mRNA levels for different MMPs are based on Patterton et al. (1995) and Stolow et al. (1996). The mRNA levels for different MMPs are plotted on different scales. Note that all MMPs have higher expression levels during tail 1 resorption than those during intestinal remodeling. Abbreviations: ST3, stromelysin-3; Col3, collagenase-3; Col4, collagenase-4, GLA, gelatinase A.

are upregulated by TH in the tail, while only ST3 is highly upregulated during intestinal remodeling (Fig. 3) (Brown et al., 1996; Damjanovski et al., 1999; Ishizuya-Oka et al., 1996; Oofusa et al., 1994; Patterton et al., 1995; Stolow et al., 1996; Wang and Brown, 1993).

In situ hybridization analyses have revealed that the expression of ST3 but not other MMPs is spatially and temporally correlated with apoptosis in both the intestine and tail during *Xenopus laevis* metamorphosis. In the intestine, ST3 is highly expressed in the fibroblastic cells underlying the degenerating larval epithelium and proliferating adult epithelial cells (Fig. 4) (Damjanovski et al., 1999; Ishizuya-Oka et al., 1996). In contrast, Col3 and Col4 have little or sporadic expression in these fibroblasts. Furthermore, the expression of both ST3 mRNA and protein is drastically activated prior to cell death and is temporally correlated with epithelial apoptosis and ECM remodeling (Ishizuya-Oka et al., 2000; Patterton et al., 1995), while Col3 and Col4 expression does not change significantly during metamorphosis (Fig. 3A) (Damjanovski et al., 1999; Stolow et al., 1996). In addition, using a human cDNA as a probe, we have also found that *Xenopus* gelatinase A (GLA) is not upregulated until stage 62 (Fig. 3A) (Patterton et al., 1995), when larval cell death is essentially complete. Such correlations argue that ST3 directly or indirectly causes specific degradation/cleavage of certain ECM components

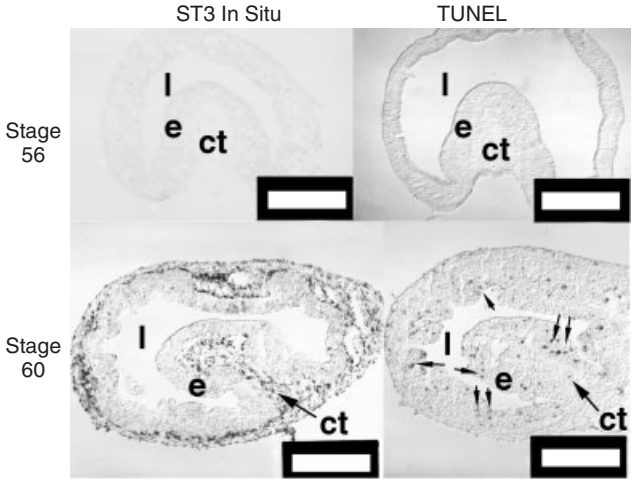


FIGURE 4. ST3 expression (in situ) is correlated with larval epithelial apoptosis (TUNEL) in the intestine. See Damjanovski et al., (1999) for more details. Abbreviations: I, intestinal lumen; e, epithelium; ct, connective tissue. The large arrows point to CT, while the small arrows point to apoptotic epithelial cells. Note that at stage 56, no ST3 expression or cell death is detected, while at stage 60, the climax of intestinal metamorphosis, ST3, is highly expressed in the connective tissue underlying the dying larval epithelial cells.

that facilitates ECM remodeling to alter cell–cell and/or cell–ECM interactions. These changes in the ECM, in turn, facilitate larval epithelial cell death. The other MMPs, especially GLA, may be involved in the removal of the ECM associated with the degenerated larval epithelium at stage 62 or later.

In the tail, all the MMP genes examined so far are highly upregulated during tail resorption in both *Rana catesbeiana* and *Xenopus laevis* (Fig. 3B) (Brown et al., 1996; Damjanovski et al., 1999; Oofusa et al., 1994; Patterton et al., 1995; Stolow et al., 1996; Wang and Brown, 1993). Interestingly, in situ hybridization analyses of Col3, Col4, and ST3 expression during tail resorption show that only ST3 mRNA is spatially and temporally associated with apoptosis (Berry et al., 1998b; Damjanovski et al., 1999). In particular, ST3 mRNA is highly expressed at stages 62 to 64 in the connective tissue underlying the degenerating epidermis and surrounding the dying muscles. In contrast, high levels of Col3 and Col4 mRNAs are present only in the connective tissue surrounding the notochord, where few apoptotic cells are detected. Thus, ST3 but not Col3 or Col4 is likely involved in the apoptotic degeneration of the tail epidermis and muscles.

Expression studies indicate that Col3, Col4, and ST3 are also expressed in other tissues/organs, including the developing limbs and remodeling

head (Berry et al., 1998a, 1998b; Patterton et al., 1995; Stolow et al., 1996; Wang and Brown, 1993). In situ hybridization shows that they are often associated with tissue degeneration events, likely involving apoptosis. Such associations may not be surprising as ECM removal is required during tissue degeneration. However, more detailed studies are needed to determine if any of these MMPs actively participates in the apoptotic process in these tissues/organs.

In addition to their involvement during larval tissue degeneration, all the MMPs are expressed at stage 62 or later in the intestine when adult tissue morphogenesis takes place (Fig. 3A) (Patterton et al., 1995; Stolow et al., 1996). In particular, both ST3 and GLA are highly expressed at stage 62 but repressed after stage 64. During this period, adult epithelial cells and cells of the connective tissue and muscles differentiate. The morphogenesis of intestinal folds also progresses through migration of the epithelial and/or fibroblastic cells, thereby establishing a crest-trough axis of the fold that is similar to the villus-crypt axis in higher vertebrates (Shi and Ishizuya-Oka, 1996). Thus, it is likely that the MMPs also participate in the establishment of new adult basal lamina to facilitate differentiation and/or cell migration.

## Functional Investigations of MMPs during Metamorphosis

Organ culture studies have provided direct evidence for the involvement of MMPs during tissue remodeling. As MMPs are extracellular proteins, it is possible to block their function in such cultures by adding inhibitors to the culture medium. For example, when tail fins are cultured in vitro in the presence of TH, they undergo resorption as during natural metamorphosis. The addition of TIMP(s) (the naturally occurring tissue inhibitors of metalloproteinases) purified from bovine dental pulp can block this TH-induced resorption (Oofusa and Yoshizato, 1991), supporting the importance of MMP activity in tail resorption.

Similarly, intestinal metamorphosis, including the apoptosis of the larval epithelial cells, can be reproduced in vitro by treating intestinal fragments with physiological concentrations of TH (Fig. 5A). In such organ cultures, ST3 expression is induced by TH as during natural metamorphosis (Ishizuya-Oka et al., 2000). Its protein level correlates with the thickening and folding of the basal lamina separating the epithelium and connective tissue as well as the death of the larval epithelial cells. In order to modulate the activity of ST3 in such organ cultures, we have generated a polyclonal antibody against the catalytic domain of *Xenopus* ST3 and shown that the antibody blocks the catalytic function of ST3 in vitro (Ishizuya-Oka et al., 2000). When this antibody is added to the culture medium of tadpole intestinal fragments, it inhibits (1) the remodeling (thickening and folding) of the

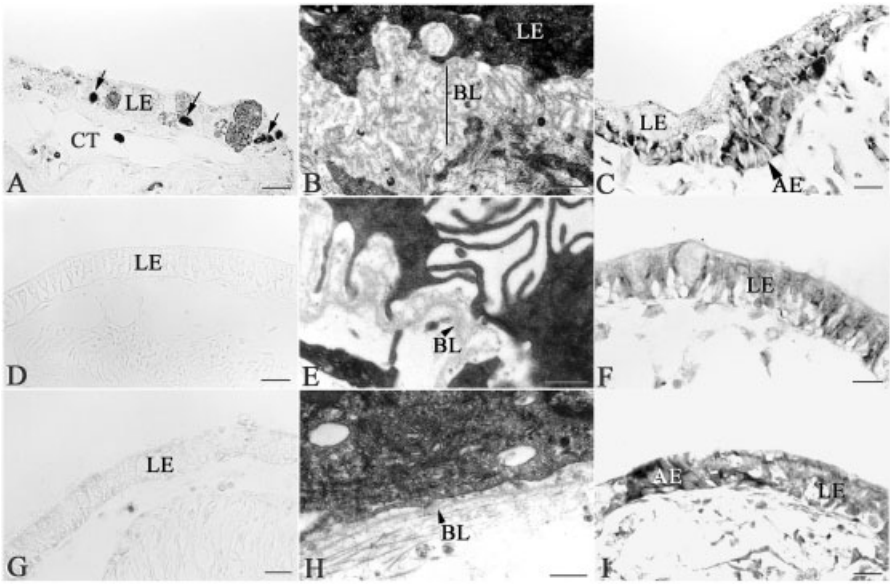


FIGURE 5. Intestinal remodeling can be reproduced in organ cultures in vitro and a blocking ST3 function inhibits this process. Intestinal explants were cultured in the presence (A–C) or absence (D–F) of TH, or in the presence of both TH and 1% anti-ST3 antiserum (G–I). (A, D, G) TUNEL assay for apoptosis in the larval epithelium. Anti-ST3 antiserum inhibits TH-induced apoptosis of the larval epithelium (LE) on day 3. Bars: 20 μm. (B, E, H) electron microscopic analyses of the basal lamina structure. Anti-ST3 antiserum inhibits TH-induced thickening of the basal lamina (BL) on day 3. Bars: 0.5 μm. (C, F, I) Methyl green-pyronin Y staining for adult epithelial cells (AE), which are stained strongly due to RNA-rich cytoplasm compared to the dying larval epithelial cells. Anti-ST3 antiserum inhibits invagination of the adult epithelial primordial (AE) into the connective tissue (CT) on day 5. Bars: 20 μm.

basal lamina (compare Fig. 5H to Fig. 5B), (2) larval epithelial cell death (compare Fig. 5G to Fig. 5A), and (3) the invagination of the adult epithelial cells into the connective tissue (compare Fig. 5I to Fig. 5C), a process critical for the adult epithelial morphogenesis (Ishizuya-Oka et al., 2000). In contrast, nonspecific antibodies or the preimune serum have no effect on TH-induced apoptosis. In addition, a synthetic MMP inhibitor, which likely inhibits the activity of many MMPs, also inhibits TH-induced apoptosis in the intestinal organ cultures (Ishizuya-Oka et al., 2000). Thus, the MMP activity of ST3 is likely responsible for its role in larval epithelial cell death and adult tissue morphogenesis.

The mechanism underlying this role of ST3 is yet unknown. Interestingly, mammalian ST3 has only weak activities toward ECM proteins but can effectively cleave α1-proteinase inhibitor (α1-antitrypsin), a non-ECM-

derived serine proteinase inhibitor (Murphy et al., 1993; Pei et al., 1994; Uria and Werb, 1998). Although ST3 may cleave ECM substrates efficiently in vivo, its ability to cleave a non-ECM substrate raises the possibility that ST3 may also affect cell behavior through non-ECM mediated pathways.

## Conclusions and Prospects

Cell death is an essential aspect of animal development ranging from *C. elegans*, *Drosophila*, to human (Jacobson et al., 1997; Jiang et al., 1997; Lee and Baehrecke, 2000; Meier et al., 2000; Wyllie et al., 1980). The apoptotic execution pathways employ several families of evolutionarily highly conserved proteins. However, the upstream signals that trigger apoptosis in development are much more complex and diverse. ECM remodeling is one such upstream signal that can facilitate or inhibit apoptosis depending on the developmental system. MMPs are critical players in the remodeling of the ECM. Many expression studies and limited in vivo function analyses have provided strong support for the participation of MMPs in regulating cell fate during vertebrate development. Our in vitro organ culture studies have demonstrated a requirement for stromelysin-3, most likely through ECM remodeling, in facilitating larval epithelial cell death and adult epithelial development during intestinal metamorphosis. The future challenge here is to confirm the findings in vivo and determine the underlying mechanisms. The recently developed transgenic methodology in *Xenopus laevis* (Kroll and Amaya, 1996) should facilitate such an endeavor. In fact, preliminary transgenic studies using the ubiquitous promoter CMV to drive the expression of *Xenopus* Col3 or ST3 or a mammalian membrane type MMP show that precocious overexpression of these MMPs leads to embryonic defects and lethality (Damjanovski et al., 2001). On the other hand, a catalytically inactive ST3 mutant has no effects on animal development. These findings are consistent with the tight regulation of MMP expression during *Xenopus* embryogenesis (Damjanovski et al., 2000). Thus, it is conceivable that by using tissue-specific and/or inducible promoters to drive MMP expression, one should be able to determine the roles of individual MMPs in ECM remodeling and cell fate determination in different organs/tissues at various development stages.

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CHAPTER

7

# APOPTOSIS IN THE IMMUNE SYSTEM

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Apoptosis is the innate mechanism by which an organism eliminates unwanted cells. Apoptotic cell death is characterized by controlled autodigestion of the cell. Cells initiate their own apoptotic death through the activation of endogenous proteases. This results in membrane blebbing, cytoskeletal disruption, and cell shrinkage. Biochemically, these alterations are associated with the translocation of phosphatidylserine to the outer layer of the plasma membrane and loss of mitochondrial function. Apoptosis also involves characteristic changes within the nucleus, with chromatin condensation and activation of endonucleases, which cleave genomic DNA into multiple internucleosomal fragments. An important feature of apoptosis is that it results in the elimination of the dying cell without induction of an inflammatory response, since the dying cell maintains the integrity of its plasma membrane.

Apoptosis is important for maintaining homeostasis in many physiological situations such as embryogenesis, tissue remodeling, and tumor formation. In the immune system, apoptosis plays an essential role in the development and maturation of T and B lymphocytes by maintaining the size of the lymphoid pool at many stages of lymphocyte maturation and activation.

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The immune system consists of a wide range of distinct cell types, each with an important role; the lymphocytes occupy the central stage, as they recognize and respond to antigens, determining the specificity of immunity. Lymphocytes differ from one another not only in the specificity of their receptors, but also in their functions. Two broad classes of lymphocytes are recognized: the B lymphocytes, which are precursors of antibody-secreting cells, and the T (thymus-dependent) lymphocytes. T lymphocytes have important regulatory functions, such as the ability to help or inhibit the development of specific types of immune responses, including antibody production and increased microbicidal activity of macrophages. Other T lymphocytes are involved in direct effector functions, such as the lysis of virus-infected cells or certain neoplastic cells.

## Apoptosis in Primary Lymphoid Tissues

An important feature of the immune system is the discrimination between self and nonself, so that the lymphocytes of each individual are able to recognize and respond to many foreign antigens, while they are normally unresponsive to the potentially antigenic substances present in the individual. Immunological unresponsiveness is also called tolerance. Antigen-dependent proliferation of a lymphocyte clone is an example of positive selection, that is, the antigen promotes growth of the cells that it activates. Under some conditions, however, contact with antigens or other stimuli results in negative selection of a responsive clone, meaning that cells in the clone selectively die. Negative selection of lymphocytes is a common event and is essential to the ability of the immune system to discriminate self from nonself (1–3). In particular, most naive T or B cells, whose antigen receptors recognize self, are thought to be killed selectively before they leave bone marrow or thymus, as a mechanism that protects the host from attack by these potentially autoreactive (self-reactive) cells. The clonal composition of the immune system is thus shaped not only by positive clonal selection, but also by active elimination of potentially deleterious clones by apoptosis (4–6).

### Death in the Thymus

Precursor cells migrate from bone marrow into the thymus, where immature precursor T lymphocytes develop into mature antigen-reactive T cells while subjected to selection (Fig. 1). Antigen recognition by T cells is self major histocompatibility complex (MHC)-restricted, meaning that T cells can recognize and respond to peptide fragments of foreign antigens only in association with self MHC. MHC molecules are expressed by many of the nonlymphoid cells in the thymus, including macrophages, epithelial cells, and dendritic cells. Interaction of maturing thymocytes with these MHC molecules in the thymus is essential in selection of the mature T cell repertoire.

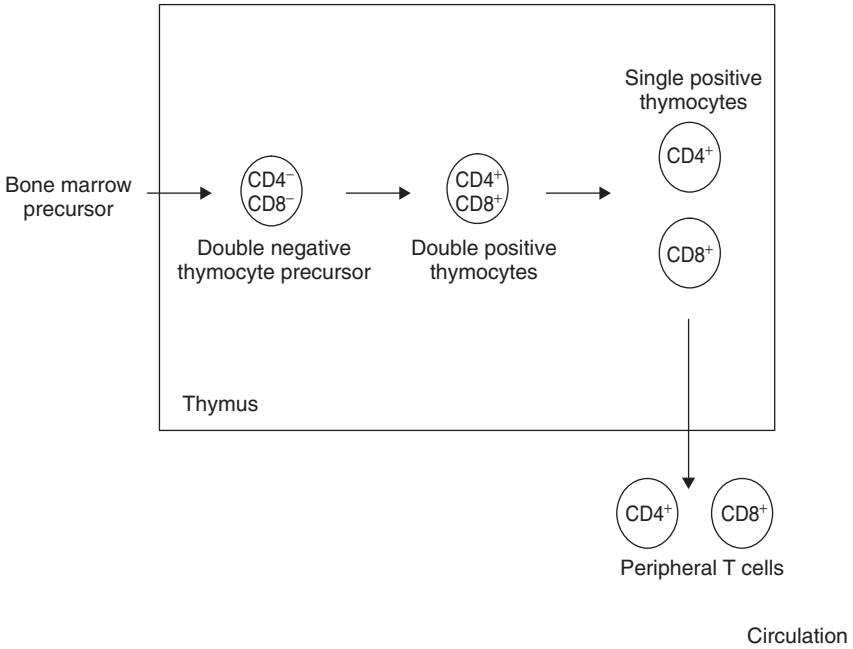


FIGURE 1. Thymic maturation of T lymphocytes. Bone marrow-derived precursors migrate through the thymus, where they become committed to the T cell lineage. Immature T cells (double negative $CD4^-CD8^-$ and double positive $CD4^+CD8^+$) are stimulated to proliferate and most progeny (more than 90%) die by selective processes.

Maturation is associated with the acquisition of high levels of T cell receptor (TCR)-CD3 complex expression and with tolerance to self antigens as a consequence of positive and negative selection. Positive selection ensures survival of immature T cells ($CD4^-CD8^-$, DN or $CD4^+CD8^+$, DP) that have rearranged the appropriate TCR; further development and proliferation of these cells depend on expression of a functional TCR β chain. Via apoptosis, negative selection deletes thymocytes with an autoreactive TCR that binds with high affinity to self antigens. The great majority of thymocytes die as a result of neglect, however, meaning that they are neither positively nor negatively selected. Death by neglect is apoptotic and occurs via exposure to endogenous glucocorticoids in thymocytes with a TCR unable to recognize self MHC. In contrast, cells bearing TCRs that recognize self MHC with moderate avidity are positively selected, whereas those that recognize MHC with high avidity are negatively selected by apoptosis (Fig. 2).

Many steps involved in apoptosis in thymus are unknown, but at least three apoptotic pathways have been identified in thymocytes: (1) a TCR-mediated pathway (6, 7), (2) a glucocorticoid-responsive pathway (8, 9), and

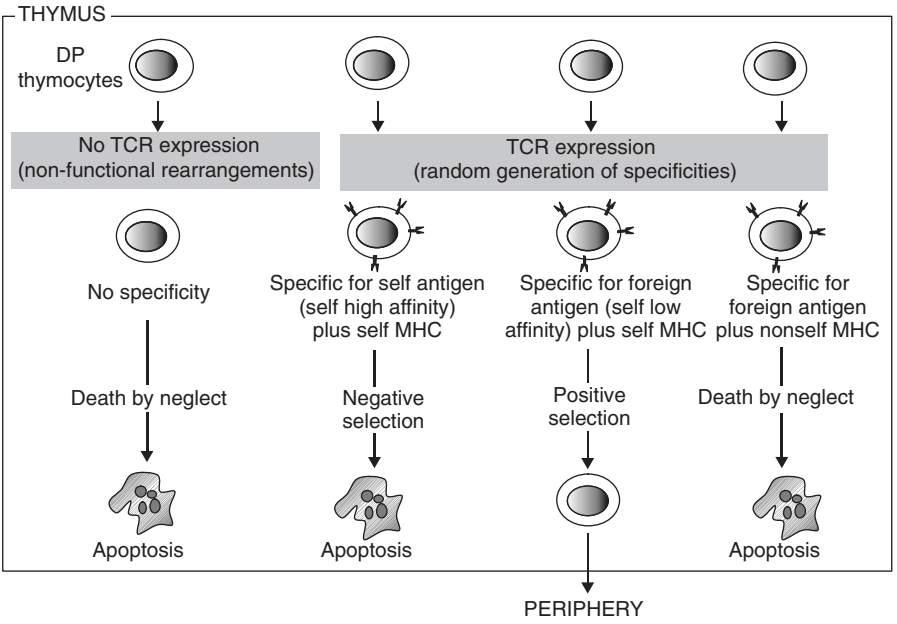


FIGURE 2. Thymic selection of T lymphocytes. Bone marrow-derived precursors with unrearranged T cell receptor (TCR) genes do not express TCRs. During intrathymic maturation, TCR gene rearrangements occur and maturing thymocytes express TCRs with random specificities. Double positive (DP, $CD4^+CD8^+$) thymocytes are then selected depending on the strength of the TCR interaction with antigen in the context of self MHC. Only those cells that recognize foreign or self antigens not present in thymus are selected for maturation, meaning that only MHC-restricted, self-tolerant T cells survive and exit the thymus to populate peripheral lymphoid tissues.

(3) a γ irradiation-sensitive pathway (10). Recent evidence suggests that these pathways can be distinguished at the molecular level. Thus, for example, γ irradiation-induced thymocyte apoptosis requires p53 and does not occur in p53-deficient thymocytes. In contrast, anti-CD3-mediated thymocyte apoptosis is p53-independent (10, 11). Bcl-2, an antiapoptotic protein, does not prevent apoptosis induced by negative selection, but is able to increase survival of immature T lymphocytes that have been positively selected (12). In contrast, other antiapoptotic proteins such as the baculovirus caspase inhibitors p35 and OpIAP are able to prevent negative selection (13, 14). Thymic hormones and cytokines secreted by thymic stromal cells, including epithelial cells, have also been described to promote T cell maturation. Among them, IL-7 requires special mention due to its central role in thymocyte proliferation (15, 16).

## Death during B Cell Development

Clonal deletion and negative selection also take place in immature B cells, leading to the elimination of self-reactive B clones. B cell development is characterized by sequential maturation steps that are guided by the surface immunoglobulin (sIg) through an incompletely characterized set of signaling pathways that appear to differ depending on developmental stage (17). Large numbers of B lymphocytes are produced in the bone marrow, but few of these cells pass the many developmental checkpoints required for tolerance and selection into the mature recirculating B cell pool. Fewer still are able to participate and expand in an antigen-driven germinal center reaction. These checkpoints are mediated by the B cell antigen receptor (BCR) and also involve positive and negative signals that control not only cell survival and death, respectively, but also regulate the Ig V(D)J gene recombinase system. Recombinase regulation maintains allelic exclusion in some circumstances, but can also modify receptor specificity in B cells through ongoing Ig gene rearrangements.

Self antigens that cross-link antigen receptors extensively cause elimination of immature B cells in bone marrow. Antigens that trigger less extensive cross-linking do not induce deletion of the B cell clones that recognize them, but produce unresponsiveness by rendering B cells anergic. In this way, when emerging sIgM on bone marrow B cells is reactive to nearby self tissue, differentiation is blocked and the cells undergo apoptosis. Nonetheless, transitional B cells may also proceed with light chain gene rearrangements (4). This developmental arrest is reversible, and a new B cell antigen receptor can be created by secondary Ig gene rearrangements that can alter BCR specificity, a process termed **receptor editing**. This mechanism thus rescues autoreactive cells from death, allowing them to alter their specificities and to mature. Unlike clonal selection, receptor editing provides a novel mechanism of immune tolerance that imposes selection at the receptor level (18, 19) (Fig. 3).

Recent studies are bringing into focus the relative roles of apoptosis and receptor editing in central tolerance. Compared to mature B cells, immature B cells have classically been considered more sensitive to apoptosis and tolerance mechanisms. Highly purified immature B cells undergo rapid apoptosis after BCR cross-linking, whereas mature B cells are more resistant to death induced by anti-BCR antibodies (20). In contrast, in unfractionated bone marrow B cell cultures, BCR ligation did not accelerate B cell apoptosis, but induced receptor editing (21–23). Bcl-2 expression promotes receptor editing in autoreactive immature B cells, suggesting that apoptosis limits the time window in which immature B cells can edit their receptors (24). Transitional B cells, which represent an intermediate stage between immature and recirculating mature B cells, are highly sensitive to antigen-induced apoptosis in vivo, whereas immature B cells edit their receptors after BCR

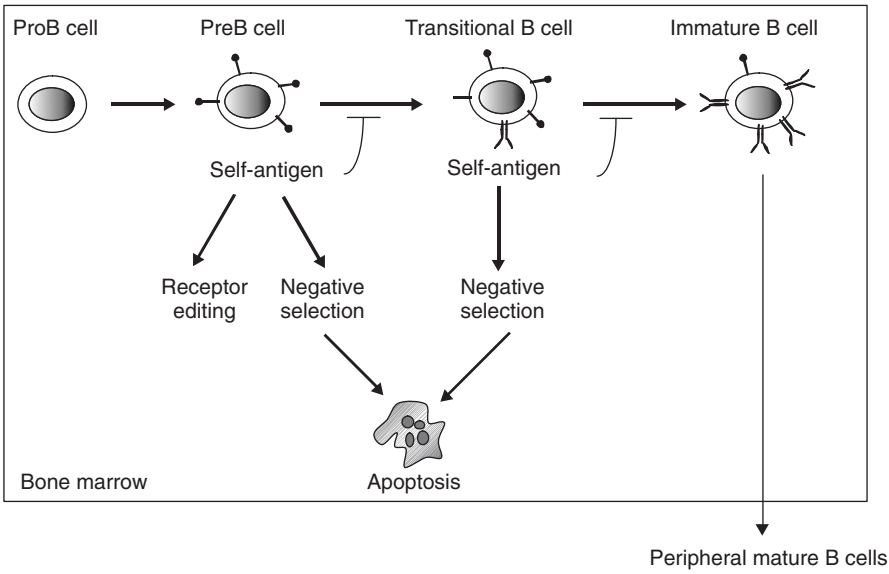


FIGURE 3. Immune tolerance in B cell development. Self antigen recognition by immature bone marrow B cells blocks progression in development and induces receptor editing. As B cells differentiate to a transitional phenotype, self antigen exposure can induce rapid apoptosis (negative selection). Also in periphery, recognition of self antigens by mature B cells can induce apoptosis.

ligation (25) (Fig. 3). Receptor editing may therefore be the preferred tolerance mechanism of newly formed B cells, but as B cells mature, they lose their ability to edit their receptors and undergo apoptosis upon self antigen binding.

Regulation of Negative Selection in Immature B Cells. Caspase and calpain activation have been implicated in BCR-induced apoptosis in immature B cells (26, 27). In fact, NOD/SCID mice reconstituted with pre-B cells expressing calpastatin (an endogenous calpain inhibitor) show deficient BCR-induced apoptosis of $IgM^+$ B cells recovered from the reconstituted mice, demonstrating the role of calpain in B cell repertoire generation (28).

CD40 signaling blocks BCR-induced deletion in immature B cells (29–34). One mechanism through which CD40 signaling prevents BCR-induced deletion of immature B cells is by blocking caspase activity (26). CD40 ligation upregulates calpastatin levels, and therefore interferes with the caspase activation pathway in immature B cells (27).

Studies with Bcl-2 transgenic mice also show that Bcl-2 expression may impair negative selection of B cells (4); nevertheless, the mechanism involved in this process is unknown. Bcl-2 overexpression counteracts BCR-triggered caspase activation in immature B cells (26), but Bcl-2 is not able to prevent

BCR cross-linking-induced growth arrest. This suggests that the pathways leading to growth arrest and apoptosis are distinct, which is supported by data showing that BCR-mediated triggering of growth arrest precedes apoptosis.

**Bcl-$x_L$** also plays a role in resistance to BCR-induced apoptosis. As for Bcl-2, Bcl-$x_L$ prevents apoptosis, but not BCR-induced growth arrest (33), indicating that signals other than Bcl-$x_L$ and Bcl-2 are necessary to promote cell cycle progression. This is supported by the observation that Bcl-$x_L$-overexpressing WEHI-231 cells, a cell line model commonly used to study immature B cell negative selection, are protected against BCR-induced apoptosis and that CD40 but not BCR signaling induces Bcl-$x_L$ expression (32, 34–36).

Bcl-2 and Bcl-$x_L$ are thought to block apoptosis by preventing caspase activation (37–41), although the mechanisms are still not clear. Cytochrome c release, which activates the mitochondrial apoptosis pathway, is prevented by Bcl-2. This pathway does not appear to play an important role in the early phase of BCR-triggered apoptosis. Caspases are essential effector molecules in apoptosis, but specific cofactor molecules are required for caspase activation and apoptosis (42, 43). Following cytochrome c release from the mitochondria, one of these adapters, **Apaf-1** (apoptotic protease activating factor-1), binds and activates caspase-9 in the presence of ATP, triggering cell death (44–46). Since Bcl-2 can regulate the subcellular localization of Apaf-1 (47), this may be one mechanism by which Bcl-2 prevents apoptosis induced by BCR cross-linking.

## Apoptosis in the Periphery

After selection, naive lymphocytes are continually released from the primary lymphoid organs into the periphery, each carrying surface receptors that enable it to bind to antigen. Antigen binding in B cells is sIg-mediated, whereas in T cells it is mediated by T cell receptors, and when accompanied by other stimuli, it can lead to B or T cell activation. Naive lymphocytes that fail to be activated die within days of entering the periphery, but those that are activated survive and proliferate, yielding progeny that may then undergo further activation and proliferation cycles. All progeny cells derived from a single naive lymphocyte constitute a lymphocyte clone. Some members of each clone differentiate into effector cells, whereas the remainder are memory cells.

### Death in Peripheral T Cells

Activation-induced Cell Death. As we mentioned above, during establishment of the T cell repertoire in thymus, thymocytes that recognize self antigens with high affinity die by apoptosis through signals induced by

their clonal receptors (negative selection), whereas thymocytes that recognize antigens with intermediate affinity, in the context of the correct MHC, leave the thymus as mature T cells. Some cells may nonetheless escape this thymic tolerance control. In addition, tolerance to self antigens present in peripheral tissues is induced only after T cells leave the thymus (peripheral tolerance). For these cells, and for those that have expanded following an immune response and must be eliminated, there is a regulated apoptotic process called activation-induced cell death (AICD) that controls immune responses. Repeated stimulation of T lymphocytes by antigens or polyclonal activators may result in death of the activated cells by AICD as a means of preventing uncontrolled T cell activation (Fig. 4).

In CD4 T cells, cell death is usually the result of paracrine interactions mediated by two molecules that are coexpressed in activated cells. These molecules, the surface receptor Fas (APO-1/CD95) and its ligand (FasL),

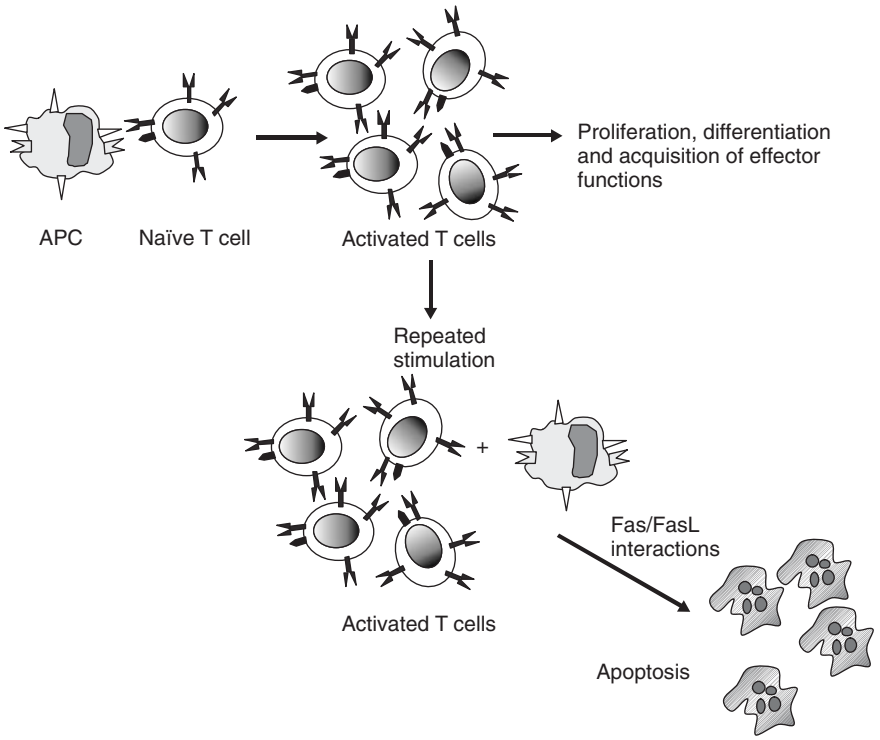


FIGURE 4. Shutdown of immune responses. A normal T cell response occurs after antigen presentation by competent antigen-presenting cells (APC), leading to T lymphocyte proliferation, differentiation, and acquisition of effector functions. Repeated stimulation of T cells results in AICD by apoptosis of activated T cells, through induction of FasL expression.

regulate the choice between cell proliferation and cell death (48–50). Fas belongs to the superfamily of the **tumor necrosis factor cell surface receptors (TNF-R)** (51, 52). FasL is expressed primarily on activated T cells. Binding of FasL to Fas, or of **TNF** to TNF-R, activates a series of intracellular cysteine proteases (caspases) that ultimately cause nucleoprotein fragmentation, apoptotic cell death, and rapid removal of dead cells by phagocytosis. Fas is the major mediator of AICD in CD4 T cells, whereas the TNF-R has a role in triggering death of CD8 T cells. AICD has an important in vivo function in T cell responses triggered by superantigens, which stimulate a large fraction of cells. In this setting, the elevated concentration produced of the interleukin IL-2 enhances FasL expression on antigen-stimulated T cells and the development of sensitivity to Fas-mediated apoptosis. IL-2 is thus both a growth factor for T cells and a feedback regulator of T cell responses.

The function of the Fas receptor and its ligand have been elucidated mainly by the finding that both molecules are mutated in mouse strains suffering from severe autoimmune diseases. *lpr* (for lymphoproliferation) mice, which lack a functional Fas receptor, as well as *gld* (for generalized lymphoproliferative disease) mice, which bear a mutant Fas ligand (FasL), exhibit various autoimmune manifestations that resemble systemic lupus erythematosus in man (53, 54). Both mouse strains produce autoantibodies and accumulate $CD4^{-}CD8^{-}$ T cells, leading to lymphadenopathy, splenomegaly, and other signs of autoimmune disorder, suggesting that Fas has an important role in regulating the immune response and maintaining self-tolerance.

In contrast, there is no convincing evidence that Fas is involved in negative selection. This assumption is consistent with relatively normal thymic architecture and correct thymic deletion of activated T cells in *lpr* mice (55, 56). On the other hand, peripheral and clonal deletion and the elimination of activated T cells are impaired in *lpr* and *gld* mice (57, 58). The Fas system is involved in both clonal deletion and elimination of activated T cells following a response to foreign antigen.

Cell Death Triggered by Growth Factor Deprivation. TCR triggering has several effects in peripheral T cells. First, primary activation of resting T cells via TCR may lead to proliferation of the T cell population. Second, in the absence of costimulatory signals, TCR triggering may cause anergy (a state of immune unresponsiveness). Finally, TCR triggering of previously activated T cells may lead to death by apoptosis, unless the cells are rescued by additional signals. There is, however, a fraction of activated lymphocytes that undergo apoptosis. This is because the local antigen and/or growth factor concentration is limiting, and the cells are deprived of essential survival stimuli. This is another homeostatic mechanism of programmed cell death independent of Fas–FasL interactions.

Target Cell Death by Cytotoxic T Lymphocytes. Cytotoxic T lymphocytes (CTL) are well-known inducers of apoptosis in target cells. CTL-induced apoptosis occurs through the action of granzymes and perforin. The mechanism used to kill by granzyme and perforin is not totally understood, but granzyme B is known to induce a G2 cell cycle kinase, cdc2, whose activation is sufficient to kill cells. Granzyme B also triggers caspase-3, linking the activation of a cell cycle kinase with that of caspases.

Mice lacking perforin or granzyme B nonetheless show low levels of CTL activity, so there is a killing mechanism distinct from perforin/granzyme-mediated death. This pathway appears to be mediated through Fas, since T cells from *gld* mice, which have no FasL, are unable to lyse Fas-positive target cells (59–63).

## Death in Peripheral B Cells

Newly produced B cells are exported from bone marrow to peripheral lymphoid tissues, from which the autoreactive population is eliminated through the process of negative selection, implicated in maintaining immunological tolerance to self. Only a subset of these new emigrants enter the long-lived pool. Positive selection mediated by basal signaling via BCR (usually by foreign antigens) may enhance B cell survival (1) (Fig. 5).

In the absence of specific helper T cells, mature B cells that encounter self antigens in peripheral tissues can be rendered unresponsive by many of the same mechanisms that cause functional anergy or death of immature B cells (64). Self-reactive B cells cannot proliferate or increase their expression of costimulators in response to self antigen, and thus cannot stimulate or respond to T cell help. Other mature B lymphocytes that have encountered self antigen become incapable of terminal differentiation into antibody secretors, by blockage of activation and germinal center entry. Self-reactive B cells may also remain functionally competent, but are unable to produce autoantibody since self antigen-specific helper T lymphocytes are deleted or anergic. Failure or breakdown of the mechanisms that control tolerance, among them apoptosis, leads to autoimmune disease. *lpr* and *gld* mice show B cell hyperreactivity associated with autoantibody production, suggesting that **Fas** also controls expansion of the B cell compartment (65, 66). During development in the bone marrow, B cells that are strongly reactive to self components are deleted, apparently by a Fas-independent mechanism (67). Surviving B cells then migrate to peripheral lymphoid organs, where they can be activated by antigens. As with T cells, Fas is involved in deletion of peripheral B cells that have been activated by self or foreign antigen. Activation of mature B cells causes Fas expression and renders the cells sensitive to Fas-mediated killing (68). In this way, **FasL** on activated T cells binds to Fas on autoaggressive B cells, which undergo apoptosis. This process is blocked in *lpr* and *gld* mice, and the B cells that escape deletion are respon-

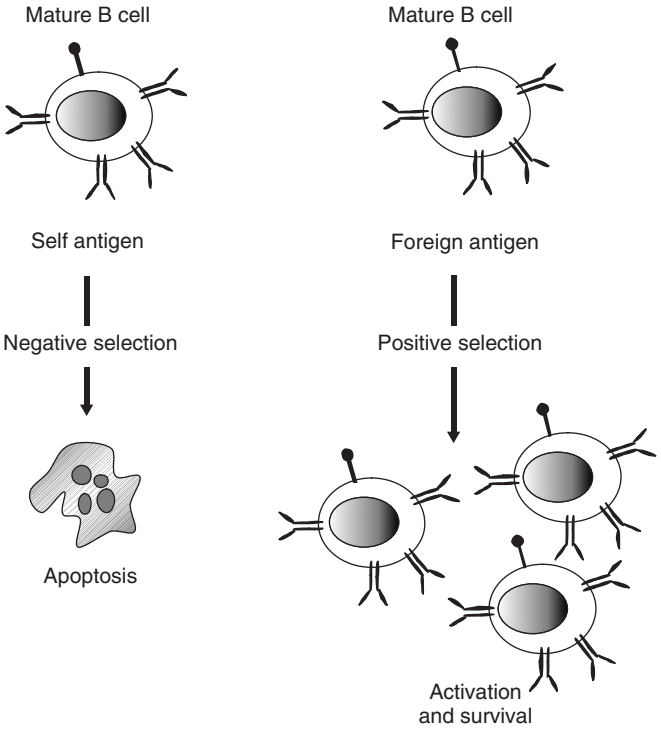


FIGURE 5. Peripheral B cell selection. Newly formed peripheral B cells that recognize self antigens are deleted by negative selection, as they cannot proliferate or respond to T cell help. Those that recognize foreign antigens are positively selected by basal signaling through the BCR, which allows them to increase expression of costimulators necessary for response to T cell help.

sible for the production of a large quantity of Ig, including autoantibodies. Fas–FasL interactions are therefore important in B cell homeostasis in the absence of self reactivity (68, 69), since activated B cells are subject to programmed cell death through the Fas pathway by cognate T cells (70–74).

Mature recirculating B cells exhibit little Fas, and BCR-mediated apoptosis is not caused by a Fas–FasL interaction (75). After activation, however, Fas expression is induced and B cells acquire susceptibility to cytotoxicity by T helper type 1 (Th1) effector cells that express Fas ligand (74, 76, 77) (Fig. 6).

Another pathway for B cell stimulation is the result of engagement by **CD40**, another TNF-R superfamily member, which interacts with its ligand to regulate induction of Fas expression and susceptibility to Fas-mediated apoptosis (74, 78, 79). In contrast, BCR cross-linking produces only a small increase in Fas expression that is not accompanied by sensitivity to Fas

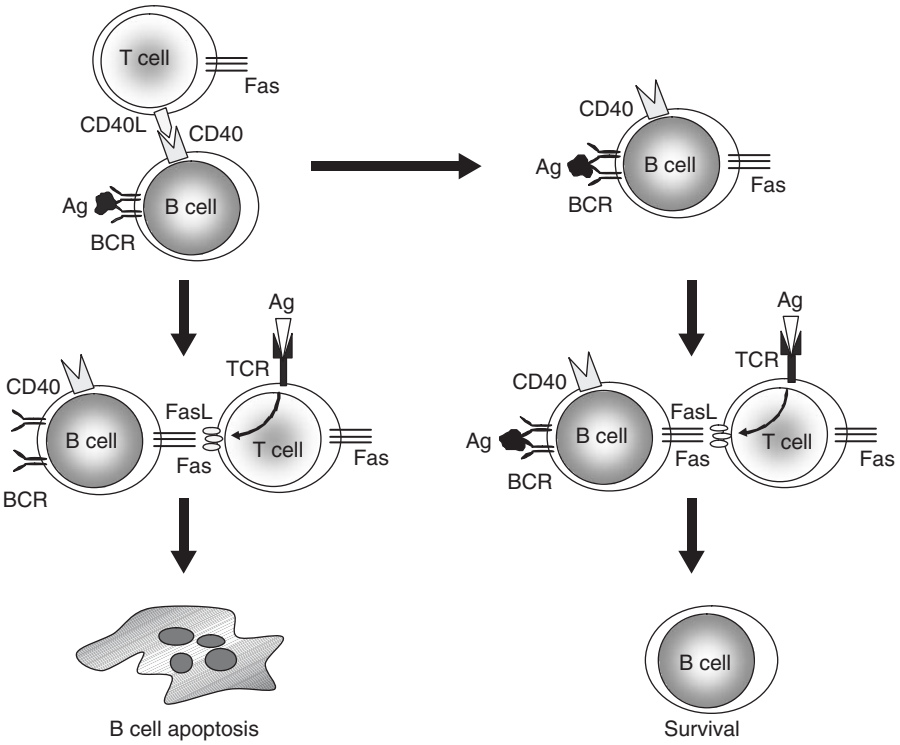


FIGURE 6. T–B cell interactions in the regulation of B cell responses. T–B cell interaction via CD40/CD40L leads to Fas upregulation in mature B cells and susceptibility to subsequent FasL-mediated apoptosis. BCR cross-linking by recognition of antigen protects B cells from Fas-mediated apoptosis.

killing. Furthermore, if B cells are stimulated through both CD40 and BCR in vitro, they become resistant to Fas-mediated apoptosis; this absence of susceptibility that is associated with BCR-mediated stimulation actually represents an active, dominant, protective process (74). Another agent able to modulate susceptibility to Fas killing is the cytokine IL-4, produced by T cells (70, 71, 80).

## Homeostasis in the Immune System

Discrimination between self and nonself is an important characteristic of the immune system, and alterations in the mechanisms that normally maintain self-tolerance can lead to a disease state called **autoimmunity**. Even when there is a failure in the selection processes that delete immature self-reactive

lymphocytes, the peripheral mechanisms should be able to maintain unresponsiveness to many self antigens. Autoimmunity may therefore be the result of a failure in peripheral tolerance, in which the anergy or deletion of autoreactive lymphocytes is altered. Encephalomyelitis and insulin-dependent diabetes mellitus (IDDM) are examples of autoimmune diseases due to T cell anergy breakdown. T cell anergy failure may occur because of abnormalities in the T cells themselves or to defects induced by inflammation, which may occur after certain infections, due to induction of tissue necrosis.

Autoimmune diseases may also result from inappropriate cell death, for instance, failure of activation-induced cell death. A defect in the **Fas** system is intimately linked to autoimmune diseases caused by the impaired removal of autoreactive lymphocytes. As already mentioned, the human autoimmune disease systemic lupus erythematosus resembles that found in *lpr* and *gld* mice, with a dysfunction of Fas or FasL, respectively (53, 54). Children with autoimmune lymphoproliferative syndrome (ALPS), also called Canale–Smith syndrome, have massive nonmalignant lymphadenopathy, hepatosplenomegaly, altered T cell populations, and other manifestations of systemic autoimmunity (81). The loss-of-function phenotype therefore indicates that Fas plays an important role in the regulation of the immune response and maintenance of self-tolerance.

Conversely, inappropriate induction of apoptosis may also lead to pathological conditions. Fas is critically involved in the progression of viral diseases such as **HIV-1** or hepatitis B virus infection, in which massive apoptosis occurs. It was shown that indirect mechanisms lead to sensitization of noninfected T cells toward AICD after HIV-1 infection (82, 83). The two HIV-1-derived proteins gp120 and Tat activate FasL expression in T cells, causing the death of uninfected T lymphocytes. This results in the continuous depletion of $CD4^+$ T cells during AIDS disease.

FasL is also responsible for the maintenance of immune privilege, which characterizes the ability of certain organs to suppress graft rejection, even when transplanted in nonmatched individuals (84). In this case, FasL acts by killing infiltrating lymphocytes of the host, preventing the resulting inflammation from destroying the tissue (85–87). FasL-mediated depletion of cytotoxic T lymphocytes may not only be beneficial, however, but may also have a role in aiding tumor cells to escape host immune surveillance. High constitutive FasL expression has been found in distinct tumor lineages, such as colon, lung, renal carcinoma, melanoma, hepatocellular carcinoma, astrocytoma, and T cell- and B cell-derived neoplasms (88–94). This suggests that the same mechanisms responsible for protecting tissues from autoimmune destruction may be used by tumors to eliminate activated lymphocytes which attempt to attack tumor cells.

Maintenance of immune system homeostasis thus depends on the strict control of cell proliferation and cell death through apoptosis, and many disease states result from errors in the regulation of apoptosis.

## Transcription Factors and Apoptosis in the Immune System: the Meeting Point?

### Effects of Steroids in Lymphocyte Cell Death

**Steroids**, especially **glucocorticoids**, as well as **retinoids**, have immunosuppressive and antiinflammatory effects and have been used in the therapeutic treatment of autoimmune diseases, leukemias, and lymphomas. Glucocorticoids have been known for many years to induce apoptosis in thymocytes (9) as well as in activated peripheral T cells (95). They are produced at high levels in the embryonic thymus and have been implicated in positive selection by antagonizing antigen-driven apoptosis (reviewed in 96). Both glucocorticoids and retinoids induce apoptosis and inhibition of c-Myc expression in Jurkat cells (97). The molecular mechanism of this inhibition has recently become clear. The **glucocorticoid receptor (GR)** binds directly to the p65 subunit of the transcription factor **NF-κB**, an important regulator of c-Myc transcription, thereby inhibiting its transcriptional activity (98). Dexamethasone induces transcription of **IκB**, an inhibitor of NF-κB, in murine T hybridoma cells and in HeLa cells (99). In addition to inducing apoptosis, steroids and retinoids also induce G1 arrest in several different primary cells and cell lines (reviewed in 100) by downregulating cyclins and CDK (101–103) as well as by upregulating CKI (104, 105).

IgM cross-linking induces NF-κB inactivation (106, 107), c-Myc downregulation (108), and cyclin kinase inhibitor p27Kip1 accumulation in WEHI-231 murine B lymphoma cells (109). p27Kip1 upregulation leads to decreased cyclin-dependent kinase 2 activity, retinoblastoma protein hypophosphorylation, G1 arrest, and apoptosis (110–112). Similar to membrane (m) IgM cross-linking, treatment of immature B cells with steroids/retinoids inactivates NF-κB, leading to c-Myc downregulation, subsequent accumulation of p27Kip1, G1 arrest, and apoptosis. Interestingly, these hormones enhance anti-IgM-induced apoptosis in immature B cells, suggesting that steroids/retinoids and mIgM cross-linking share a common signal transduction pathway. All these steroids/retinoid-induced effects can be prevented via CD40/CD40L signaling, which rescues cells from G1 arrest and apoptosis (113).

This common signal transduction pathway may at least partially explain why steroids and retinoids enhance anti-BCR-mediated apoptosis in B lymphoma cells, in contrast to thymocytes and T cell hybridomas, in which they antagonize AICD (114, 115).

### Apoptotic Functions of p53

One of the most important links between the proliferation and cell death machinery is the tumor suppressor p53, which promotes cell cycle arrest or apoptosis in response to DNA damage or a strong oncogenic stimulus to proliferate (116). Recent evidence shows that oncogene expression and DNA

damage use different mechanisms to induce p53-dependent apoptosis (117). The importance of oncogene-dependent p53 death is illustrated by the fact that most tumors show disruption of either p53 or an upstream activator of this pathway, the p19ARF tumor suppressor. Expression of oncogenes such as those encoding E2F1, c-Myc, E1A, or oncogenic versions of Ras results in the accumulation of **p19ARF** (118–121), the product of an alternative reading frame (ARF) of INK4 (122). This induction occurs through several mechanisms (118, 121), and p19ARF acts in different ways to promote p53 stabilization and function (123–126). Oncogene expression in p19ARF null cells results in strongly attenuated p53 induction (although it is not lost completely) and these cells do not undergo apoptosis (120, 121). Normal cells that survive the senescence crisis or c-Myc overexpression generally show mutations in either p53 or p19ARF, but not in both, consistent with the idea that these genes function in the same death-promoting pathway (121, 127). The fact that p19ARF null cells show normal p53 induction following exposure to DNA-damaging agents (127), and that cells lacking elements of the DNA damage-dependent p53 pathway are normal with respect to oncogene-dependent p53 induction (128), indicates that DNA damage and oncogene expression induce p53 through separate pathways.

The best understood activity of p53 is its ability to function as a transcription factor that can induce or repress expression of a large and growing number of genes, although less well-defined transcriptionally independent activities of p53 have also been described (129). The importance of transcriptional regulation by p53 has been demonstrated in many studies, in particular by the generation of mice or embryonic stem cells in which replacement of the wild-type protein by a transcriptionally inactive mutant p53 resulted in loss of cell cycle arrest and apoptotic functions (130, 131). Transcriptional activation of the p21WAF1/CIP1 cyclin-dependent kinase inhibitor plays a key role in the induction of cell cycle arrest by p53, but there does not appear to be a similar critical apoptotic target. Possible mechanisms include transcriptional activation of the proapoptotic Bcl-2 family member Bax (132, 133), the generation of reactive oxygen species (134), and transcriptional upregulation of death receptors such as CD95/Fas/APO-1 or DR5/KILLER (135–138). In fact, p53 can directly engage each of the major apoptotic pathways in the cell, stimulating both death receptor signaling and mitochondrial perturbation, including cytochrome c release. Loss of caspase-9 or Apaf-1 renders mouse fibroblasts resistant to p53-dependent apoptosis (139). It was recently shown that Apaf-1 is a direct transcriptional target of p53, independent of the pRb pathway, suggesting that p53 might sensitize cells to apoptosis by increasing Apaf-1 levels (140, 141).

## The Role of Rb in Apoptosis

The retinoblastoma protein **pRb** and its close relatives p107 and p130 have key roles as negative regulators of cell proliferation. Consistent with this role,

the pRb pathway is deregulated in most human cancers (reviewed in 142, 143). Although loss of pRb results in hyperproliferation, it also results in p53-dependent and -independent apoptosis (144). The best-characterized targets for the pRb family members are the **E2F transcription factors** (145, 146). The E2Fs are best known for their role in regulating the timely expression of genes required for DNA replication and cell cycle progression. Concurring with their role as essential downstream targets of pRb, ectopic expression of several of the E2Fs results in hyperproliferation and apoptosis (147–156). E2F1-induced apoptosis is potentiated by the presence of wild-type p53, and it is believed that loss of pRb results in apoptosis as a consequence of increased E2F activity (144–146). This model is supported by data showing that loss of E2F1 expression suppresses apoptosis and hyperproliferation in Rb-deficient mouse embryos (157). A few E2F target genes have been described that might mediate E2F-induced apoptosis; most prominent among these are p14/p19ARF and p73 (120, 155, 156). Although ARF appears to play a role in potentiating E2F-induced apoptosis through stabilization of p53, ectopic ARF expression results in cell cycle arrest rather than apoptosis (158), suggesting that other genes involved in apoptosis are regulated by E2F. In contrast to ARF, ectopic p73 expression results in apoptosis (159); however, it is at present unclear whether the p73 levels achieved as a consequence of E2F1 expression are sufficient to affect cell proliferation.

A direct link between deregulation of the pRb pathway and apoptosis is provided by the finding that Apaf-1 expression is directly regulated by E2F1 (140), and that Apaf-1 is required for E2F-induced apoptosis (Fig. 7).

## Rel/NF-κB Transcriptional Regulators Are Critical for Cell Division, Cell Survival, and Cell Death

Several studies reflect the fact that NF-κB can regulate expression of distinct proapoptotic and antiapoptotic programs in different cell lineages, at different developmental stages of a single lineage, and/or in response to different extracellular signals.

Members of the Rel/NF-κB family of transcriptional regulators are activated by a broad range of signals including cytokines, mitogens, free radicals, and stress signals. These transcription factors control the expression of a wide range of genes implicated in cytokine signaling, immunity, and development. Studies using mice that lack members of the Rel/NF-κB gene family have identified their essential roles; for example, B lymphocytes require the NF-κB proteins p50 and p65, as well as c-Rel for mitogen- and antigen-receptor-induced proliferation (160). Interestingly, p50 is needed for the survival of quiescent B cells, whereas c-Rel is more important for the survival of activated B cells. Impaired B cell survival is probably due to reduced induction of pro-survival Bcl-2 family members such as A1/Bfl1 or Bcl-$x_L$

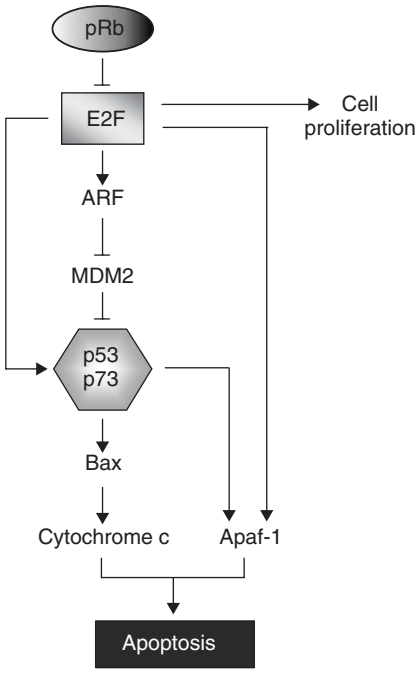


FIGURE 7. Model for the regulation of E2F-induced apoptosis. E2F directly regulates the transcription of ARF, p73, and Apaf-1. The increased ARF or p73 levels result in the transcriptional activation of Bax (and other p53/p73 target genes), and the subsequent triggering of cytochrome c release from mitochondria. Simultaneously, Apaf-1 levels are increased, and the active Apaf-1/procaspase-9 holoenzyme can stimulate apoptosis.

(161). Expression of the antiapoptotic proteins Bcl-2 or A1 permits survival of Rel/NF-κB-deficient B cells, but does not restore cell proliferation. Collectively, these results demonstrate that Rel/NF-κB transcription factors are critical regulators of both cell proliferation and cell survival. The data also indicate that Rel/NF-κB proteins control these two processes by activating and/or repressing distinct sets of genes.

Mice lacking p65 die during development due to abnormal hepatocyte apoptosis (162), a lethality that can be prevented by TNF deficiency (163). As TNF/TNF-R1 signaling is essential for hepatocyte proliferation after partial liver resection, it appears that p65 (and possibly other Rel/NF-κB family members) control expression of proteins that determine whether this signaling triggers cell proliferation or apoptosis.

T lymphocytes require c-Rel and, to a lesser extent, other Rel/NF-κB-related genes for mitogen- or antigen-induced proliferation (164). Surprisingly, these molecules appear to be unnecessary for T cell survival, although

they are obligatory for B cell survival. Rel/NF-κB may be involved in AICD of T lymphocytes, as it stimulates Fas ligand expression (165), which is essential for this process (166). This suggests that Rel/NF-κB proteins have different effects on apoptosis in different cell types. NF-κB is not required for p53-dependent thymocyte apoptosis, but is a critical positive regulator of at least one p53-independent pathway of programmed cell death in DP thymocytes. In fact, NF-κB mediates anti-CD3-triggered apoptosis in wild-type DP thymocytes by downregulating expression of the Bcl-$x_L$ antiapoptotic gene. As Bcl-$x_L$ is the predominant antiapoptotic gene expressed in DP thymocytes (167, 168), it is not surprising that its downregulation would predispose these cells to apoptosis. Such a model is also consistent with previous studies showing that constitutive expression of a Bcl-$x_L$ transgene in DP thymocytes protects them from anti-CD3–mediated apoptosis (168).

## Other Antiapoptotic Transcription Factors

In addition to NF-κB, other transcription factors act by activating antiapoptotic genes. The TAFII105 subunit of TFIID transcription factor is essential for the activation of antiapoptotic genes in response to TNF-α, serving as coactivator for NF-κB. This was further demonstrated after antisense expression, which sensitized cells to TNF-α-mediated cytotoxicity (169).

In contrast to NFATp and NFATc, NFAT4 is expressed preferentially in DP thymocytes. Mice lacking NFAT4 have a thymus defect characterized by increased apoptosis of DP thymocytes. Increased sensitivity to apoptosis may reflect heightened sensitivity to TCR-mediated signaling. These mice also have impaired production of Bcl-2 mRNA and protein; NFAT4 thus has an important role in the successful generation and survival of T cells (170).

## c-Myc: A Double-edged Sword

The c-Myc protein, encoded by the c-myc proto-oncogene, is a potent inducer of both cell proliferation and apoptosis (171, 172). The proapoptotic property of c-Myc is shared with other mitogenic oncoproteins such as E1A (173) and is thought to act as a built-in restraint to the emergence of neoplastic clones (174–176). Substantial evidence indicates that c-Myc-induced apoptosis and mitogenesis are discrete downstream programs, neither of which is necessarily dependent on the other. Activation of the molecular machinery that mediates cell-cycle progression is not required for c-Myc-induced apoptosis (177). Furthermore, c-Myc-induced apoptosis in serum-deprived fibroblasts is inhibited by survival factors such as insulinlike growth factor 1 (IGF-1) that exert little, if any, mitogenic effect on such cells (178). The apoptosis suppressor Bcl-2 likewise inhibits c-Myc-induced apoptosis (179–181), with no measurable effect on the oncoprotein's mitogenic activity (180). Finally, experiments in mice in which c-myc has been specifi-

cally deleted in B cells show that B cells are resistant to FasL-mediated apoptosis due to lower Fas expression (182, 183).

One intriguing possibility is that c-Myc does not itself induce apoptosis, but rather acts to sensitize cells to other pro-apoptotic insults. Indeed, c-Myc expression has been shown to sensitize cells to a wide range of mechanistically distinct insults such as serum or growth-factor deprivation (171, 172), nutrient privation (173), hypoxia (184), p53-dependent response to genotoxic damage (172), virus infection (185), interferons (172, 186), TNF (187), and CD95/Fas (188), many of which have no obvious effect on cell proliferation. This role as a sensitizer to different apoptotic stimuli implies that c-Myc must act at some common node in the regulatory and effector machinery of apoptosis.

## DIO-1: The Direct Connection

DIO-1 (death inducer-obliterator-1) was first identified using a differential display approach in WOL-1 pre-B cells that were induced to undergo apoptosis by IL-7 starvation (189). Its predicted amino acid sequence showed transcriptional activation domains, a canonical bipartite nuclear localization signal (NLS), a PHD finger, and a lysine-rich carboxy-terminal region. DIO-1 mRNA and protein levels were upregulated soon after apoptotic induction by various stimuli, including IL-7 removal, dexamethasone or γ-interferon addition in WOL-1 cells, sIgM receptor cross-linking in WEHI-231 cells, or c-Myc activation under serum-free conditions in the absence of p53 expression in MEF(10.1)Val5MycER cells. Overexpression of the gene product in cells or misexpression in chick limb induces massive apoptosis in the absence of any apoptotic stimulus. DIO-1-induced cell death can be inhibited by Bcl-2 overexpression or incubation with the general caspase inhibitor z-VAD-fmk. These results suggest that DIO-1-induced apoptosis requires caspase activation. In addition, DIO-1 upregulates procaspase-3 and -9 protein levels, and increases the activity of their mature forms. This direct link between activation of an apoptosis-regulating gene and caspase activation provides an explanation for the rapid apoptosis induction observed after DIO-1 expression (190).

Overexpression of a DIO-1 deletion mutant lacking both NLS failed to induce cell death, linking its lack of lethality to an inability to translocate. More interesting, this mutant prevented B cells from undergoing growth factor withdrawal-induced apoptosis, behaving as a dominant negative mutant. In fact, the DIO-1 protein is located in the cytoplasm of healthy cells, but is rapidly translocated to the nucleus in the presence of an appropriate apoptotic stimulus (190).

## Conclusions

Apoptosis is the mechanism of programmed cell death that controls the generation of B and T cell repertoires and regulates immune responses. This process must be highly regulated, since deregulation of apoptosis can lead to several pathological situations. Failure of apoptosis may lead to autoimmune disease, probably due to the presence of self-reactive clones, as well as to an increase in inflammatory responses, since activated lymphocytes generated after an immune response may not be correctly eliminated. Uncontrolled activation of the apoptotic pathway may prevent the induction of immune responses required for elimination of pathogens. Apoptosis thus controls T and B cell numbers during development and following an immune response by maintaining the balance between cell death and cell survival, ensuring the homeostasis of the immune system.

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CHAPTER

8

# CELL DEATH IN NEURONAL DEVELOPMENT AND MAINTENANCE

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The mammalian nervous system is composed of highly complex assemblies of neurons, which interact with each other in very specific patterns, and also make connections with virtually every other structure in the body. The CNS of a human brain is estimated to have 10 to 12 billion neurons, and each neuron can make as many as 1,500 synaptic connections. Given this enormous complexity (and not forgetting the presence of 100-fold more glial cells that are also of neurogenic origin), it is not surprising that a stochastic rather than deterministic model of apoptotic neural cell death in development has been adopted during evolution. Overall, it is estimated that between 30 and 50% of all the neurons born die during development (Oppenheim et al., 1992).

In the past few years, a vast amount of evidence has accumulated showing that the intrinsic and the extrinsic (death receptor) pathways of apoptosis play key roles in developmental neuronal cell death, especially when neurons are establishing connections with their targets. The questions of interest currently are therefore primarily to identify the signaling mechanisms that give rise to apoptosis, to understand how apoptosis is directed

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so that only selected structures are eliminated, and whether the survival strategies enacted during development can be applied to prevent neuronal cell death in injury and neurodegenerative disorders. More recently, there has been increasing interest in the roles of apoptosis during the earlier developmental periods of neurulation, neurogenesis, and neural migration, especially since the targeted disruption of Apaf-1 (Cecconi et al., 1998; Cecconi and Gruss, 2001), caspase-9 (Yoshida et al., 1998), or caspase-3 (Kuida et al., 1996) was found to cause immense hyperplasia during the earliest stages of brain development, and gross brain malformations in the more mature brain. The identity of survival factors that regulate these early cell deaths, their source, and their signaling mechanisms are almost unknown. An interesting correlate of these studies is the increased evidence for the requirement for DNA recombination and repair during neurogenesis (Chun and Schatz, 1999; Frank et al., 2000; Lee et al., 2000). Given the considerable amount of information that has accrued in these areas, and the questions that still remain regarding mechanisms of survival in the more mature brain, we have chosen here to focus on two aspects of neuronal death during development where immense progress has been made recently: (1) the evidence for apoptosis during early neural development, and the mechanisms that regulate it and (2) new insights into the regulation of neuronal death in the established nervous system.

## Role of Apoptosis during Neural Tube Closure and Neural Tube Defects

Neurulation is the earliest process where apoptosis has been described as being essential. During neurulation, the dorsal ectoderm is induced to form the neural plate, which folds to form the neural tube. Newborn cells differentiate and migrate out into the tube's periphery, giving rise to the CNS (brain and spinal cord). Other cells derived from the hinge region adjacent to where the neural tube closes form the neural crest; these cells migrate along the body axis and coalesce into ganglia to form the peripheral sensory, autonomic, and digestive nervous systems. Lack of neural tube closure causes neural tube defects (NTD), leading to conditions such as exencephaly and spina bifida. Weil and coworkers (1997) examined the process of neural tube closure in the thoracic region of explanted chick embryos where closure first begins and noted the presence of many acridine orange staining cells in the neural tube and neural folds, elevated regions where the hinges meet and seal the neural tube. When they inhibited nominal caspases using zVAD-fmk or Boc-D-fmk, closure of the neural tube was prevented but zFA-fmk (a cathepsin inhibitor) had no effect. The essential involvement of apoptosis in this process is also demonstrated by the forebrain overgrowth in the fog

mutant mouse, which possesses a hypomorphic allele of Apaf-1 and, among other symptoms, shows NTD (Honarpour et al., 2001).

How might this apoptosis be regulated? NTD are found in a high proportion of female mice in which the p53 tumor suppressor gene is disrupted (Armstrong et al., 1995; Sah et al., 1995), raising the possibility that p53 mediates the apoptotic death during this period. However, it appears that the defect caused by the absence of p53 is sexually dimorphic and not universal as only a tiny proportion of p53 null male mice show NTD. The causes of NTD are very heterogeneous and only a small proportion of NTD are due to direct defects in apoptosis (reviewed by Harris and Juriloff, 1999). Recently, the involvement of p53 in apoptosis and NTD has been examined in a different way. Pani and colleagues (2002) investigated the causes of apoptosis and NTD in the Sp/Sp mice (which are Pax3 null) by crossing the mice with p53 null mice. They observed that apoptosis in the neural tube and NTD are absent in double Sp/Sp × p53 null mice and that NTD was reduced by 55% in Sp/Sp mice by pifithrin-α, a nominal inhibitor of p53-induced apoptosis (Komarov et al., 1999). It is suggested that Pax3 functions to suppress p53 protein expression (but not its mRNA), thereby inhibiting apoptosis and hence implying that p53 is causing NTD through excessive apoptosis. But since Sp/Sp mice show increased levels of p53 compared to wild-type embryos, it is still not clear whether apoptosis in the normal neural tube is mediated by p53. Extensive p53-dependent cell death was also specifically detected at E10.5 in Mdm4 mutant embryos, but here too the constitutive activity of p53 is particularly high (Migliorini et al., 2002). Thus, if p53 is utilized to mediate apoptosis during normal development of the neural tube, its effects are clearly dependent on other factors. Moreover, these factors must be kept under tight regulation if excessive apoptosis, as well as the inhibition of apoptosis, leads to NTD.

Two groups described severe NTD in JNK1/2 double knockout mice (but not in the JNK3 null mice, although the JNK3 gene is expressed only in the CNS) (Haydar et al., 1999; Kuan et al., 1999; Sabapathy et al., 1999). Interestingly, apoptosis decreased at E9.5 in the neural folds, roughly equivalent to the developmental period studied by Weil and coworkers in the chick embryo, and consistent with a proposed role for JNK and its downstream target c-Jun in inducing neuronal apoptosis (reviewed by Ham et al., 2000; Herdegen and Waetzig, 2001; Weston and Davis, 2002). However, by E10.5 there was massive apoptosis in the forebrain that led subsequently to its extensive degeneration. Interestingly, there was no degeneration in other nascent brain structures such as the mid- and hindbrain, demonstrating how regionally specific and context-dependent these apoptotic inducers can be. Extrinsic pathways may also play a role in NTD, as mice null for one of the tumor necrosis factor (TNFα) receptor-associated factors, TRAF 6, also show NTD. However, apoptosis has not been examined during neural tube closure

itself and a reduction in apoptosis is observed in later development (Lomaga et al., 2000). Interestingly, the penetrance of this mutation is also dependent on genetic background, as are those of Apaf-1, caspase-9 and -3 (see below).

If apoptosis is necessary for neural tube closure, what sets the limits on the amount of cell death in the neural tube? Little work addressing this question has been performed in mammals. Work by de la Rosa and de Pablo (de la Rosa and de Pablo, 2000; Rubio et al., 2002) in the chick embryo suggests that unprocessed pro-insulin expressed locally induces selective survival by upregulating the expression of the chaperone and heat shock-cognate protein HSc70, consistent with the notion that the survival of all cells requires an active suppression of cell-autonomous cell death pathways (Raff, 1992). Antisense constructs directed against either pro-insulin or HSc70 mRNA were found to exacerbate apoptotic death throughout the neural tube. The further role of heat shock proteins in preventing neuronal death is suggested by recent data from C. Woolf's lab (Costigan et al., 1998; Lewis et al., 1999) which has showed that sensory and sympathetic neurons expressing higher levels of the chaperone Hsp27 were more likely to survive during the PCD period. The involvement of pro-insulin (or the insulin-like signaling pathway) in shaping the brain in mammals is supported by the differences in brain sizes when the expression of IGF-1/2, their receptors, or binding proteins is perturbed (reviewed by D'Ercole et al., 2002). However, there are no gross morphological defects or NTD reported so the effects are quite subtle. The main effect is on oligodendrocytes and the process of myelination. In the IGF1 null mouse, for example, although brain size is reduced, this effect takes shape postnatally. There appears to be a selective loss of some types of neurons (Beck et al., 1995), but the major loss is of "white" matter, the myelinated axons and oligodendrocytes. In the IGF1 receptor null mouse there is also decreased size of the brain and spinal cord (and of the whole body), but the number and organization of the neurons in the CNS appear to be normal (Liu et al., 1993), although increased crowding of the neurons is observed. There thus remains a great deal to learn about the mediators of apoptosis during neurulation, and in particular, there is a need to define the mechanisms that spare most of the cells from apoptosis.

## Apoptotic Death during Neurogenesis in Neuroblast and Immature Neuron Populations: Epistatic Relationships between Bcl-2 Family Members, Apaf-1, and Caspases

Neurogenesis is the period that occurs after neurulation when almost all the neurons and glial cells inhabiting the adult brain are born in the region of

the neural tube closest to the ventricles, the ventricular and subventricular zones. Depending on the polarity of cell division, neuroblasts and glioblasts become committed to their respective fates, begin to withdraw from the cell cycle, and migrate out to form layers (as in the cortex, cerebellum, and retina) or nuclei (collections of cells that serve a common function). As mentioned already, striking brain malformations are observed in Apaf-1 (Cecconi et al., 1998; Cecconi and Gruss, 2001), caspase-9 (Yoshida et al., 1998), and caspase-3 (Kuida et al., 1996) knockout mice, consistent with the remarkable amount of hyperplasia observed during neurogenesis. Ceconni and Gruss (1998) further showed that Apaf1, caspase-9, and caspase-3 are epistatically related in the apoptotic pathway during neurogenesis in the cortex and retina—there is no activation of caspase-3 without caspase-9, and no activation of caspase-9 or -3 in the absence of Apaf-1 (reviewed by Cecconi and Gruss, 2001). A major feature in these knockouts is the extended proliferation and survival specifically of neural progenitor cells, defined by their expression of some proneuronal markers, and their relatively normal distribution in the different brain areas. Hence, it is a differentiated type of cell that is affected, arguing against random proliferation as being the cause of hyperplasia.

Roth and colleagues have further analyzed the epistatic relationships between the Bcl2-family members Bcl-xL, Bax, Bcl2, and Bid, and Apaf-1, caspase-3, and caspase-9. Using Bcl-xL null mice (Motoyama et al., 1995) as their pivot, they limited their analysis to immature telencephalic cortical neurons that are early postmitotic cells, since Bcl-xL null mice die at E13.5 probably due to defective hematopoiesis (reviewed by Kuan et al., 2000; Roth and D'Sa, 2001). Crossing these different lines of mice, they found that the excessive neuronal death in Bcl-xL null mice—in which caspase-3 was excessively activated—was almost completely rescued by crossing the mice with mice null for Apaf-1, caspase-9, caspase-3, or Bax. Although Bcl2 null mice showed no gross abnormalities at this stage, double Bcl-xL/Bcl2 knockouts showed more widespread signs of apoptosis. However, Bid null mice showed no obvious developmental abnormalities, arguing against the involvement of the Fas/TNF family of death receptors in developmental PCD in the brain during this period (Leonard et al., 2001). Furthermore, no rescue of Bcl-xL$^{-/-}$ neurons was obtained by crossing the mice to p53$^{-/-}$ mice (Klocke et al., 2002), arguing against an essential role for p53 in regulating cell death after neurulation. This contrasts with a notable role for p53 in mediating the cell death of similarly staged neurons in response to DNA damage (D'Sa-Eipper et al., 2001). Here, nonepistatic relationships were found between p53 and Bax, Apaf-1, or caspases, consistent with nonapoptotic mechanisms being available to execute death in apoptosis-execution-deficient cells, which may involve the mitochondrial release of the pro-apoptotic protein AIF (Cregan et al., 2002).

Numerous studies have shown that Bax plays a key role in regulating the final number of many types of postmitotic neurons, including motoneu-

rons, sensory neurons, sympathetic neurons (Deckwerth et al., 1996; White et al., 1998), retinal ganglion neurons (Mosinger Ogilvie et al., 1998), and cerebellar Purkinje neurons (Fan et al., 2001). There are increased numbers of mature neurons in each of these populations in the Bax knockout mice. However, despite the epistatic relationship between Bcl-xL and Bax in the embryonic brain, Bax deletion by itself seems to have only minor effects on the programmed cell death during early development. The overall appearance of the brains of adult Bax null mice is amazingly normal, as is their apparent behavior. Even in Bax/Bak double knockouts, where tissues that were hitherto unaffected in the single knockouts are now malformed (no digit formation, no vaginal opening), there is some increase in the number of dividing neuroblasts found in the ventricles, but certainly no massive alteration of cortical lamination or differentiation (Lindsten et al., 2000). In vitro, however, postnatal cortical neurons from Bax null mice are extremely resistant to trophic factor deprivation and DNA damage (A. Wong and A. Tolkovsky, unpublished observations), in keeping with an important role for Bax later in development. Since the absence of Bax also prevents AIF-mediated death induced by DNA damage (Cregan et al., 2002) in early (E14.5) embryonic cortical neurons, it may be that none of the insults in the in vitro studies match the conditions that give rise to PCD during development. Curiously, none of the morphological features that are so striking in the brains of caspase-3 null mice were found after the 129X1/SvJ strain in which they were developed was exhaustively bred into the inbred C57Bl/6 strain for 9 to 12 generations (Leonard et al., 2002). However, just one backcross into the 129 strain was sufficient to regenerate the malformations. Thus, strain-specific modifiers and/or compensatory caspase activation/inhibition mechanisms (Zheng et al., 2000; Troy et al., 2001) must be taken into account when trying to draw conclusions about the importance of apoptosis in mouse brain development from global knockouts (see also the discussion below). At the same time, these experiments provide the possibility of identifying such modifiers if they exist. Whether there is less cell death during neurogenesis in these and other mutants that affect apoptosis in the C57Bl/6 strain remains to be investigated.

## Why Is There so Much Cell Death during Neurogenesis: Is It the Result of Conflict or DNA Damage?

In a striking and initially controversial study by J. Chun's group, apoptosis demonstrated by labeling DNA ends using the ISEL (in situ end labeling) technique suggested that over 50% of all the newborn neuroblasts die in the mouse CNS (Blaschke et al., 1996, 1998). These deaths were apoptotic as they

were greatly reduced in caspase-3 knockout mice (Pompeiano et al., 2000). Thomaidou and colleagues (1997) compared the number of mitotic figures and that of TUNEL-positive nuclei (another DNA-labeling technique) in the neurogenic ventricular zones of rats and calculated that cell death affects one in every 14 cells (7%) produced by dividing ventricular zone cells at embryonic day 16 and about one in every 1.5 cells (40%) produced in the subventricular zone of newborn rats. Although this extent of cell death was known to occur in peripheral neuron populations during the period of target innervation (Oppenheim et al., 1992), this result was unexpected in the CNS since neurogenesis was thought to be target-independent. Strikingly, recent work using spectral karyotyping in diving neuroblasts and interphase FISH analysis in early postmitotic populations has identified approximately 33% of neuroblasts as being aneuploid (Rehen et al., 2001). One probable fate is cell death as there is decreased aneuploidy observed in the adult cortex relative to the embryonic cortex. Moreover, this cell death may be controlled, since FGF2 added to cultures of cortex derived from these layers reduces the amount of aneuploidy apparently by preferential loss of those cells with greater than one chromosome gained or lost. However, aneuploidy may not necessarily augur death as some aneuploid neurons survived into adulthood as postmitotic neurons. An alternative, but not mutually exclusive, possibility is that mature neurons may also undergo distinct processes, resulting in aneuploidy.

Also of note is the observation that cortical layering is not affected by the migration of aneuploid neurons to their final destinations. Interestingly, although it might be expected that changes in brain size reflect changes in the incidence of apoptosis, changes in brain size provide little indication of the existence of excessive, or lack of, apoptosis as evidenced by two mouse models (different from those in which IGF-1 or IGF-2 was expressed transgenically beginning at birth; D'Ercole et al., 2002): In one model, the pro-apoptotic kinase GSK-3 [whose activity can be suppressed by phosphorylation by survival-related kinases such as Akt and by the Wnt signaling pathway (Woodgett, 2001)] was expressed transgenically in a constitutively active form; in the other model, a constitutively active, stable from of β-catenin (which is normally destabilized and targeted for degradation by phosphorylation by GSK-3) was transgenically targeted to neuronal precursor cells. Expression of activated GSK-3 promoted a 20% decrease in brain size without any signs of excessive apoptosis, the reduction in volume being ascribed to decreased neuronal soma size and concordant increases in neuronal density (Spittaels et al., 2002). In contrast, expression of β-catenin in neural precursors caused a huge expansion in neuronal numbers and an increase in brain size, but this was due primarily to a massive increase in the surface area of the cortex through creation of numerous deep folds (as found in human brains) (Chenn and Walsh, 2002). However, migration and lamination appear to be normal. What supports the survival of these numerous

neurons is not clear. The authors expected apoptosis to have decreased given the increased number of surviving cells (Haydar et al., 1999), but instead observed a 2-fold increase in the number of TUNEL-positive cells. This might not be too surprising, given a probable mismatch between the increased neuron numbers and availability of survival factors from neighboring cells that, as discussed above, presumably control the number of apoptotic cells during development. However, if we consider that caspase-3 or -9 and Apaf-1 knockout animals show a different set of morphological abnormalities involving increased neurogenic zones and ectopic growth while these brains seem to be organized normally, it is tempting to speculate that β-catenin might have actually suppressed cell death in some of the precursor population, thus accounting for the rather small increase in TUNEL positive cells compared to the final cell number achieved. Transgenic overexpression of β-catenin in the postmitotic adult mouse brain (under the control of the PrP promoter) does not cause any cancers (Kratz et al., 2002), arguing against it being very mitogenic.

The problem with studying cell death during cortical development is that 3 to 4 weeks may elapse between the period of time when neurons are generated and the time when they project to their final targets so it is not possible to study the signals that regulate apoptosis in different types of identified neurons in the same age of the animal. This problem is somewhat overcome in the retina. In the retina of newborn mice and rats, fully differentiated postmitotic neurons coexist with neuroblasts and newly postmitotic neurons. Retinal ganglion cells (RGC) are already fully differentiated, and project to their targets, such that retinal excision causes deprivation of target-derived factors and RGC apoptosis within 24 hours. At the same time, the ventricular layer is still generating neuroblasts, while the neuroblastic layer (NBL) contains immature postmitotic but undifferentiated neurons that are in the process of migration to form the other layers of the retina. These processes continue according to schedule when the retinas are cultured in vitro. Linden and colleagues compared the sensitivities of these different classes of neurons to agents that disrupt cell cycle and cell death. For example, Rehen and coworkers (1999) found that anisomycin, which inhibits protein synthesis, inhibited the apoptotic death of RGC and the proliferating neuroblasts while enhancing the apoptotic death of the newly postmitotic neurons in the NBL. They suggest that during migration and initial differentiation in the NBL, the apoptotic machinery may be blocked by suppressor proteins, thus allowing recently formed postmitotic cells to find their final positions and differentiate while being protected from apoptosis. Partially selective inhibitors of cyclin-dependent kinases (cdks) (some of which also inhibit JNKs and other kinases) also revealed differential sensitivities to the induction of apoptosis in the RGC, neuroblasts, and newly postmitotic neurons migrating in the NBL. Taken together with the studies of Roth and colleagues, and Slack and colleagues, it appears that immature

neurons that have recently emerged from the cell cycle are most vulnerable to cell death during development. Whether this is because they are migrating away from—or even changing—their requirement for survival factors (Davies, 1994) is not clear.

The neurotrophic factors that support survival of some of the cell types in the chick retina have been partially characterized. One group (Karlsson et al., 2001) suggests that NGF is produced by the more differentiated horizontal cells in the retina and is used to support these cells' survival in an autocrine fashion. Another group (Gonzalez-Hoyuela et al., 2001), studying retinal ganglion cell development, found that NGF, which promotes the death of RGC neuronal precursor cells via activation of the p75NTR receptor when they are in the last M phase (Frade et al., 1999), is produced by RGC that have completed migration to their final layer. This NGF is proposed to diffuse so as to kill the incoming p75NTR-expressing RGC while protecting the NGF-generating RGC from death through TrkA, thus autoregulating their cell numbers. Killing lots of migrating RGC depleted the eventual numbers of other neuron types in the postmitotic retina, suggesting that the number of neuroblasts born is not limitless. In their discussion, Gonzalez-Hoyuela and colleagues also remark that they observed that NT-3 promotes RGC genesis also by binding to p75NTR, consistent with the multifarious effects mediated by signaling via p75NTR (for a review, see Roux and Barker, 2002); the abnormal cell cycle of trigeminal sensory neuronal precursors in $NT3^{-/-}$ mice was also noted (El Shamy et al., 1998). The p75NTR can thus signal for mitosis, survival, and death independently of Trks depending on which neurotrophin is bound, and shift its signaling mechanisms to additional paradigms when cognate Trk receptors are expressed and stimulated. These combinations expand enormously the repertoire of effects that are induced by neurotrophins. Although Fas/TNF family death receptors may also show complex signaling patterns, and the p55 TNF receptor, at least, has more than one ligand, their impact on brain development is not as apparent as that of p75NTR. One wonders whether there are additional receptors in the nervous system that behave like p75NTR.

Given the enhanced sensitivity of newly differentiated neurons to cell death over the proliferating and postmitotic populations, the idea promoted first by Ucker (1991) that "physiological cell death occurs through a process of abortive mitosis" is still a favored hypothesis among some researchers. This theme was later adopted and applied to neuronal cell death by Heintz (1993) and Rubin et al. (1994). The idea is that an aberrant attempt at S phase entry in differentiating neurons leads to apoptosis as a result of conflicting growth control signals. Greene's lab has done the most to examine this hypothesis (reviewed in Liu and Greene, 2001). From this body of work, it is proposed that the retinoblastoma (Rb) protein is the key link to death, as Rb null embryos exhibit gross neurological (but also hematopoietic) defects and there is extensive apoptosis in the nervous system. Moreover, the expres-

sion of several classes of cdk inhibitors, or DN forms of cdk4/6, which permit Rb to function as a repressor of the E2F family of transcription factors, inhibited apoptosis in model systems (reviewed by Ferguson and Slack, 2001). Frade (2000) also suggested that in the chick retina unscheduled entry into the cell cycle precedes killing by NGF through p75NTR. To reexamine the role of Rb in neuronal apoptosis, and eliminate the contribution of hematopoiesis to embryonic lethality, Slack and colleagues (Ferguson et al., 2002) interbred mice with a floxed Rb allele with mice in which Cre was knocked into the *Foxg1* locus (Foxg1 promotes gene expression that is restricted to the telencephalon in the cortex; Hebert and McConnell, 2000). They found that although the Rb null progenitor cells divided ectopically, they were able to generate postmitotic neurons, the mice survived until birth, and there were no signs of excessive apoptosis. Although it might be argued that the Rb-related proteins p107 and p130 replaced the roles of Rb in survival, this should have also happened in the global Rb knockout mouse, and indeed it has been hypothesized that muscle cells and neurons from Rb null mice are unable to undergo terminal differentiation because p107 and p130 replace this function of Rb (Schneider et al., 1994). There are too few groups testing the "signal-conflict" model to decide on its value; perhaps this is because we now prefer to understand the function of genes and are reluctant to give processes names that may prove to be stumbling blocks to understanding function.

## Recent Insights into Neurotrophin Actions and Neuronal Maintenance

The developing nervous system was the first system in which it was noted by Hamburger (1975) and Levi-Montalcini (Levi-Montalcini and Angeletti, 1968) that the final number of cells is determined by PCD. Hamburger and Levi-Montalcini were also the first to propose that the size of neuronal populations was controlled by the limited supply—or access to the supply—of trophic factors from these targets. Although their main research was devoted to spinal motoneurons and peripheral neurons, it is now clear that similar dependencies occur in the postmitotic CNS, for example, in the retina, where retinal ganglion neurons that project to the superior colliculus depend, at least in part, on as yet poorly defined trophic factors for their survival (reviewed in Cellerino et al., 2000). In the cerebellum too, granule neurons that synapse onto pyramidal neurons depend on the latter for their survival (as these neurons die in the Lurcher mouse in concordance with the death of pyramidal neurons, but fail to die to a large extent in mice in which Bax was deleted by targeted disruption; Doughty et al., 2000). There remain however vast numbers of structures in the CNS whose natural period of death both before and after target innervation has not been charted, for

example, many of the deep nuclei, the striatum, the thalamus, the hippocampus. This is a difficult area that cries out for further investigation.

An interesting twist with regard to the neurotrophic hypothesis has arisen from studies of muscle innervation by incoming motoneurons in zebrafish. During innervation of the muscle target, two neurons—the CaP/VaP pair—start out being equivalent, but only one normally lives. However, the neuron that ends up dying does not die because of a limiting amount of neurotrophic factors or available space at the muscle target; rather, it seems that the first neuron to reach and grow past an intermediate cell type becomes CaP, and by doing so induces the intermediate cells to produce a factor that disables the second neuron, which is now identified as VaP, from arriving at the final target. Hence, ablation of the intermediate cells allows both CaP and VaP to arrive at the final target and live (Eisen and Melancon, 2001). The type of death of VaP, and the mechanisms of apoptosis in zebrafish in general, are just beginning to be understood (Cole and Ross, 2001).

An interesting feature in the developing CNS that has become appreciated recently is the reciprocal nature of source and target survival. Thus, axonal transport of trophic molecules occurs not only in a retrograde manner (from the target to the cell body), but also in an anterograde fashion, with release at the nerve terminals. In the visual system, the RGC neurons that project to the superior colliculus (or tectum in chick) also promote tectal neuron survival as the blockade of anterograde transport or electrical activity in the nerve (electrical activity is required for neurotransmitter and neurotrophin release in the CNS; Canossa et al., 1997) increases apoptosis in the target (Cellerino et al., 2000). NT-3 appears to be the major anterograde signal responsible for this effect as endogenous NT-3 was shown to be transported anterogradely by tracing the course of radiolabeled NT-3 (von Bartheld and Butowt, 2000), and intraocular NT-3 injection prevented the anterograde degeneration of tectal neurons that occurs after injection of pertussis toxin into the eye (a process thought to ablate endogenous NT-3 levels without affecting anterograde transport; von Bartheld et al., 1996). In the rat, similarly, intraocular administration of BDNF can reduce cell death in the target structures (Spalding et al., 2002). Reciprocal relationships have also been noted between Schwann cells and sensory spinal DRG neurons. A Schwann cell forms an intimate one-to-one relationship with its axon, which it proceeds to myelinate; those cells that fail to find a free axon fail to receive the appropriate signal and die by apoptosis (Jessen and Mirsky, 1998). This signal derived from the axon is neuregulin (one of multiple splice forms from the NRG-1 gene), the ligand for the ErbB3 receptor (in a heterodimer with ErbB2). In ErbB3 receptor null mice there is a complete lack of Schwann cell precursors and thus mature Schwann cells in peripheral nerves (Riethmacher et al., 1997). Detailed analysis of developing embryos shows that at E12.5, the DRG in the ErB3 knockout mice is much the same as the wild-type embryo, but by E14.5 there are 70% fewer neurons and by E18.5

82% fewer. A similar loss is noted for spinal motoneurons, although at a later stage of development. Axonal control of glial survival is also seen with oligodendrocytes in the developing CNS [reviewed in (Barres and Raff, 1999)]. How much of this reciprocity occurs in other systems is still unclear, although the retrograde effects of neurons on other facets of incoming innervation, such as the number of synaptic inputs, have long been known (e.g., Lichtman and Purves, 1980). Moreover, there is now clear evidence for BDNF being able to stimulate electrical activity in target neurons, just as if it were a bona fide neurotransmitter (Blum et al., 2002).

Notwithstanding these new kinds of regulatory roles ascribed to neurotrophic factors, the availability of knockouts for all the neurotrophins (NGF, BDNF, NT3, NT4/5) and their receptors (Trks A, B, and C, p75NTR) has provided incontrovertible evidence that the absence of death suppression by these factors has devastating effects on survival maintenance of the nervous system (reviewed by Snider, 1994; Bibel and Barde, 2000; Ernfors, 2001; Huang and Reichardt, 2001). Although single knockouts are sufficient to decimate specific populations of peripheral neurons, it is necessary and sufficient to reduce the gene dosage of two different Trk gene allelles to promote widespread cell death in the developing CNS (Minichiello and Klein, 1996). The dominance of neurotrophic support over the availability of specific cell death mechanisms (Du and Montminy, 1998) can be see in animals lacking caspase-3 or caspase-9 expression, where the same final number of motoneurons is found in the spinal cord of mutant and wild-type mice, although this death occurs with some delay and its mechanism is non-apoptotic and nonnecrotic (Oppenheim et al., 2001). As mentioned above, motoneurons are spared in Bax knockout mice but in trigeminal sensory neurons, even the lack of Bax expression could not replace the loss of NGF or TrkA expression (although sympathetic neurons were completely rescued in the NGF or TrkA null backgrounds by Bax deletion) (Middleton and Davies, 2001).

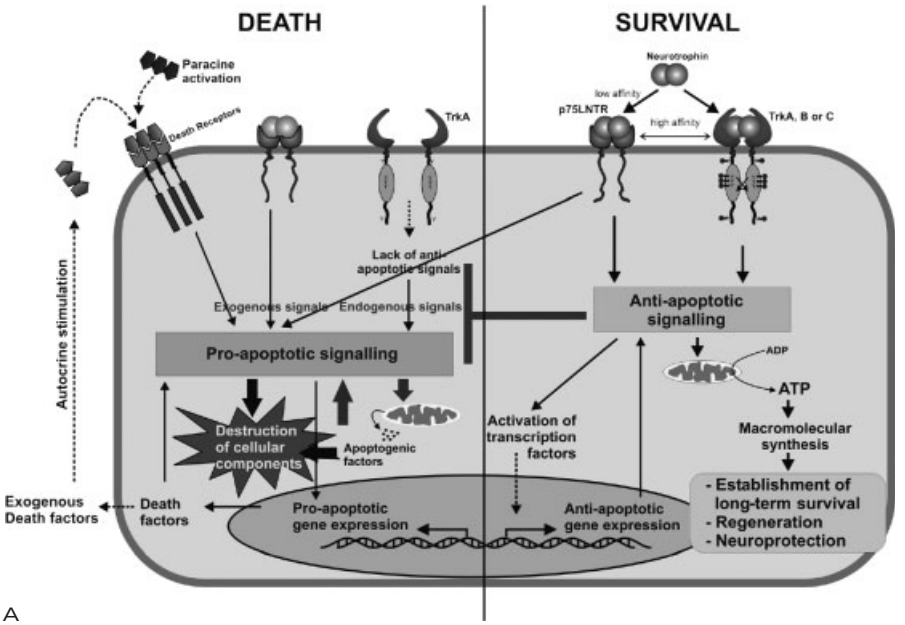
The mechanisms by which neurotrophins support survival during development seem to be similar to those described in other cell types, namely through a dominant, Ras-, PI 3-kinase-, and PKB/Akt-dependent pathway whose function is to suppress cell death signaling upstream of the point of Bax activation (reviewed recently by Brunet et al., 2001; Miller and Kaplan, 2001). Interestingly, although the Ras/ERK pathway appears to have little impact on survival in the context of neurotrophic factor deprivation, this pathway in the same neurons is used to suppress death induced by DNA damage (Anderson and Tolkovsky, 1999; Hetman and Xia, 2000). The targets of survival-signaling pathways are cell-type- and cell-context-dependent, and not all these targets are found in every neuronal type. A summary of some of these pathways and their interrelationships is given in Fig. 1. Since the PKB/Akt pathway is predominant during the development of many neuronal classes, we shall describe what is known about this pathway in a

little more detail. There is evidence that PKB/Akt can suppress a JNK-dependent pro-apoptotic pathway by phosphorylation of the upstream MLK family member, ASK-1, phosphorylation of Rac1 (Kwon et al., 2000; Kim et al., 2001), and/or by rearranging the JNK scaffold protein JIP-1 (Kim et al., 2002). Further pro-apoptotic targets phosphorylated and inactivated by PKB/Akt include the pro-apoptotic BH3-domain protein BAD and the Forkhead transcription factor FKHRL1 (Brunet et al., 2001), whose functions are abrogated by their binding to 14-3-3 proteins in the cytoplasm, and the kinase GSK-3 (Cross et al., 1995) mentioned above. Inhibiting death signaling, though, is not enough; there are also pathways that are required to nurture the neurons to maintain them alive (Fletcher et al., 2000; Xue et al., 2001). Pro-survival pathways induced by Akt phosphorylation include activation of the survival-inducing transcription factor CREB (Du and Montminy, 1998; Lonze and Ginty, 2002). However, although overexpression of activated Akt1 can inhibit neuronal death in response to the absence of trophic factors (Philpott et al., 1997; Crowder and Freeman, 1998; Virdee et al., 1999; Hetman and Xia, 2000; Xue et al., 2000), inhibition of the PI 3-kinase signaling pathway both pharmacologically and using dominant negative inactive Akt1 does not always culminate in apoptosis as rapidly as that which occurs by removal of the survival factor itself, suggesting that additional survival pathways operate, at least in sympathetic neurons (Philpott et al., 1997; Virdee et al., 1999; Tsui-Pierchala et al., 2000). Additional survival signals activated by neurotrophic factors include the transcription factor NFκB, whose activity in mouse CNS neurons was demonstrated recently using a transgenic reporter, and shown to be involved in the survival of CNS neurons in the brain (Bhakar et al., 2002). In a few select cases, but not all types of neurons, upregulation of Bcl-xL and IAP-family proteins have also been observed in response to NGF (Wiese et al., 1999).

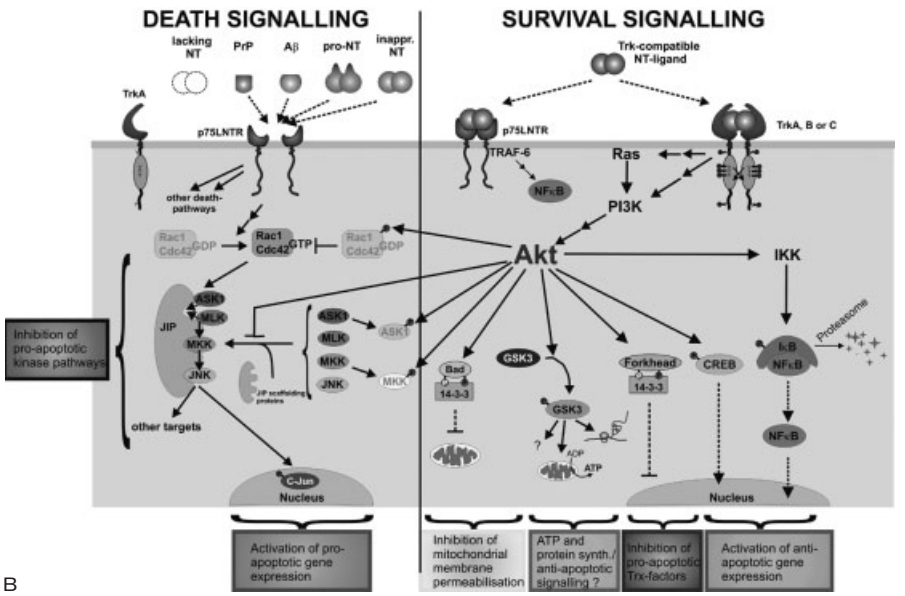
Many of the mediators of survival and death during development are being proposed based on culture studies. Transcription factors such as c-Fos, c-Jun, and NFκB are notorious for being activated by cellular stresses or injury. Hence, while uncovering survival and death pathways that are activated by neurotrophic factors is important in the context of trying to negate the effects of injury or disease, establishing their relevance to early development will require techniques that interrupt signaling in vivo in a cell- and developmentally-restricted manner without causing extraneous signals (although these types of studies are not without their own problems).

Another interesting and controversial area of research is the role of death receptors in apoptosis during brain development. p75NTR has been mentioned already as a receptor that can promote apoptosis (as well as survival) in the presence (or absence) of specific ligands in the appropriate developmental context. In addition to numerous in vitro studies, in vivo studies show that transgenic overexpression of the p75NTR intracellular domain promoted the death of several neuronal populations (Majdan et al., 1997). In

addition, TrkA suppresses apoptosis in part via its suppression of p75NTR signaling, since a dysfunctional (although incomplete) p75NTR knockout [p75NTR$^{exon3}$ (Lee et al., 1992; Naumann et al., 2002)] delayed the loss of some of the sympathetic neurons that would have been dead in TrkA$^{-/-}$ sympa-




A




B

thetic ganglia (Majdan et al., 2001). For receptors of the Fas/TNF family, there are less clear roles during development. A cursory observation of the brains of *lpr* (Fas-defective) and *gld* (Fas-ligand defective) mice, as well as $Bid^{-/-}$ mice, does not reveal dramatic abnormalities. However, Fas has been implicated in the death of embryonic motoneurons by Raoul and colleagues (1999, 2002) who also propose a motoneuron-specific death signaling

FIGURE 1. (A) The role of neurotrophins in regulation of neuronal survival and death. Neurotrophins bind to two types of receptors: The pan-neurotrophin receptor p75NTR binds all neurotrophins (nerve growth factor [NGF], brain-derived neurotrophic factor [BDNF], neurotrophin-3 [NT-3] and NT-4 [NT-4]) with low affinity, whereas the receptor tyrosine kinases of the Trk family (TrkA, B, and C) bind them in a specific manner. TrkA binds preferentially NGF, TrkB binds BDNF and NT-4, and TrkC binds NT-3. Although the Trk receptors are often quoted as high-affinity receptors, they require association with p75 to form these high-affinity binding sites (Dechant, 2001). In the absence of neurotrophin-mediated signaling through the Trk receptor, the low-affinity pan-neurotrophin receptor p75 elicits a pro-apoptotic signaling pathway. Interestingly, p75 can initiate pro-apoptotic signals either in the absence of any neurotrophins or when it is ligated with neurotrophin ligands that do not stimulate the corresponding Trk receptor. Furthermore, pro-forms of neurotrophins (that do not bind to Trks) or unrelated proteins (Prion protein, Aβ) are able to activate p75 (Dechant and Barde, 2002). Pro-apoptotic signaling leads to the upregulation of death effector proteins (e.g., BH3-only proteins) that enhance pro-apoptotic signaling, or it even induces expression of soluble death factors (e.g., FasL), which are secreted and act by paracrine stimulation on other death receptors. Eventually, this pro-apoptotic signaling culminates in the release of apoptogenic factors from the mitochondria that commit the cell to death through a caspase- and nuclease-dependent destruction of proteins and genomic DNA. Activation of the Trk receptor initiates a signal transduction pathway that inhibits pro-apoptotic signaling, as well as affecting cellular growth and differentiation, and it can also provide protection against various insults. (B) The PI3K/Akt pathway is an example of a multifunctional suppressor of pro-apoptotic signaling and a mediator of growth signals. The Trk-stimulated effector Akt antagonizes pro-apoptotic signaling at various levels. It inhibits activation of the JNK pathway by phosphorylation and inactivation of signaling constituents (Rac1, Ask1, MKK-4), and by (a phosphorylation-independent) disruption of the assembly of a signaling module involving JIP and the JNK pathway (Kwon et al., 2000; Kim et al., 2001, 2002; Park et al., 2002). Phosphorylation of the BH3-only protein Bad and the transcription factor FKHRL1 (Forkhead) sequesters them into a cytoplasmic complex with 14-3-3 proteins, preventing them from participating in mitochondrial permeabilization or induction of pro-apoptotic gene products, respectively (Brunet et al., 1999; Datta et al., 1997). On the other hand, transcription factors are activated that lead to expression of antiapoptotic proteins (Bcl-2, Bcl-xL, IAPs, etc.) (Brunet et al., 2001). Furthermore, Akt-dependent phosphorylation inactivates glycogen synthase kinase-3b, which results in an increase of glucose utilization and transcriptional activation, and possibly also inhibits other pro-apoptotic signaling (Cohen and Frame, 2001).

pathway in apoptosis, which integrates canonical Fas signaling via caspase-8, and a special pathway that is mediated by Fas activation of Daxx, which in turn activates p38 kinase, which in turn activates nNOS expression and activity. Readers who wish to learn more about neurotrophic factors, the mechanisms of survival signaling, how and in what form these signals are transported to cell bodies from targets, and the different roles emerging for secreted, mature, and pro-NGF (reminiscent of the specialized role found for proinsulin, perhaps) are referred to some of the many excellent recent reviews on these topics (e.g., Huang and Reichardt, 2001; Neet and Campenot, 2001; Airaksinen and Saarma, 2002; Dechant and Barde, 2002; Ginty and Segal, 2002; Hempstead, 2002).

## Adult Neurons Switch Their Mechanisms of Trophic Factor Dependence

The brain never ceases to develop. Thus, it is valid to ask how mature long-lived neurons that have been spared from cell death during formation of the nervous system maintain their integrity during subsequent periods. This question is interesting as there are numerous stresses and potentially harmful stimuli that impinge on neurons throughout their life. In the sympathetic and sensory peripheral nervous systems, where this question has been studied most, there are substantial shifts in neurotrophic requirements from extrinsic to intrinsic mechanisms of protection. The progress of maturation to gain independence from exogenous support by NGF can be mimicked in vitro by long-term culturing of NGF-dependent neurons in the presence of NGF, thus allowing the biochemical investigation of the underlying mechanisms. Although NGF withdrawal from long-term cultures of DRG and SCG neurons still resulted in c-Jun-phosphorylation [which can be pro-apoptotic (Ham et al., 2000)], Bax translocation and subsequent cytochrome c release, events committing the young neurons to death, did not occur in the mature neurons (Easton et al., 1997; Vogelbaum et al., 1998). Furthermore, NGF-deprived long-term cultures of SCG neurons were resistant to apoptosis induced by cytoplasmic injections of cytochrome c (Putcha et al., 2000), whereas 6-day cultured neurons lost their initial resistance to cytochrome c during NGF deprivation (Deshmukh and Johnson, 1998; Neame et al., 1998). Further shortening of the apoptotic response time after cytochrome c injection can be induced by severing neuronal connections to their (axonlike) neurites, showing that there is wide scope for the regulation of apoptosis in response to cytochrome c (Fletcher et al., 2000). Little is known about this aspect of regulation in other classes of neurons. During the process of becoming NGF-independent, DRG neurons increased their expression of Bcl-xL relative to that of Bax (Vogelbaum et al., 1998). Furthermore, these mature neurons became less dependent on PI 3-kinase signals for NGF-mediated survival (Klesse and Parada, 1998; Vogelbaum et

al., 1998). However, PI3-kinase was deemed necessary for NGF-independent survival of adult SCG neurons in culture (Orike et al., 2001). How active PI 3-kinase is maintained in these neurons is still not known. The same authors also proposed that the survival of NGF-independent SCG neurons is Bcl2/Bcl-xL-dependent, as antisense-Bcl-2 or Bcl-xL induced apoptotic cell death in culture. Yet mature Bcl-2 null SCG neurons still achieved NGF independence (Greenlund et al., 1995). Careful monitoring of the expression levels of all pro- and antiapoptotic Bcl-2 family members will be necessary to rule out the possibility that the redundant regulation of other Bcl-2 proteins (e.g., Bcl-xL, Bcl-w, A1, Diva, or Mcl-1) accounts for growth factor independence in the Bcl2 null neurons. Interestingly, another Bcl2 null mouse showed gradual loss of neurons in postnatal life after the natural cell death period but hardly any deficiency during the PCD period (Michaelidis et al., 1996; Cellerino et al., 2000).

## Summary

Although there have been huge advances in understanding neuronal cell death during development, several questions remain to be answered. The description and temporal map of PCD for several types of neurons are still not complete. The signals that give rise to apoptosis need to be better understood. The survival factors that interact with apoptotic pathways so that only selected structures are eliminated remain to be identified, and their mechanisms of death suppression during early development need to be elucidated.

The value of studying mechanisms of PCD during development are clear; by understanding the survival strategies enacted during development, it may be possible to devise similar strategies to prevent neuronal cell death in injury and neurodegenerative disorders. There is now burgeoning interest in the use of embryonic and adult neural stem cells to replace neurons in disease. The roles of apoptosis in controlling neural stem cell numbers in the adult brain, and the survival factors that counterbalance mitosis and apoptosis to maintain this population are just beginning to be deciphered. Given the pace of current research, and recent advances in analyzing complex genomics, transcriptomics, and proteomics (and soon metabolomics), it is likely that the new tools emerging will also help unfold the answers to these complex problems. As in the pioneering days of Santiago Ramon y Cajal (1928), however, the best neuroscience research still, and always will, require painstaking attention to detail.

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CHAPTER

9

# CELL TURNOVER: INTESTINE AND OTHER TISSUES

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In recent years, our knowledge of the cellular and molecular mechanisms that regulate cell turnover in epithelial tissues has grown significantly. In particular, the intestine has been an organ system that has yielded important information about cell survival and cell death controls in epithelial tissues. The late 1970s and 1980s were characterized by experiments that defined the classical methodologies, still used today, for assessment of proliferation and apoptosis in vivo using light and electron microscopy-based techniques. These experiments established the concept of stem cells and cellular hierarchies, with their differential capacities for proliferation, differentiation, and apoptosis. The late 1980s and early 1990s saw the emergence of chimeric and transgenic animal models, which have since had a major role in defining the regulation of cell death and cell proliferation in epithelial tissues. In particular, the ROSA 26 mouse (in which stem cells and early progenitor cells express β-galactosidase) and the targeted expression of genes in the intestinal epithelium, using specific promoters such as for FABPI (fatty acid binding protein of the intestine), have been important developments. Gene knockout animals have also been developed during this time and have been particularly useful in defining genes important in the response to cell

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injury. Our understanding of epithelial biology has grown also with our understanding that cell types do not exist in isolation but exist as part of a dynamic, interactive environment within the tissue as a whole. Consequently, cells such as fibroblasts, lymphocytes, and commensal and pathogenic microorganisms are appreciated as being important in directing epithelial cell fate. Given the vast amount of new data, this review will concentrate on the intestine as a model tissue.

## Definition of Key Terms

Crypt. This is the proliferative unit within the small and large intestine. All epithelial cell production takes place within the crypts, which are flask-shaped pits within the intestinal mucosa.

Stem Cells. Multipotent cells within a tissue that are capable of self-renewal and that have the potential to generate all the differentiated, cell phenotypes within that tissue. In the intestine crypts are monoclonal, that is, all the cells are descended from one *master stem cell*.

Cell Lineages. A defined pathway of cellular descent from a single stem cell. A single lineage may branch one or more times with each branch having its own defined fate, that is, to fulfill a particular differentiated function within the tissue. Lineages of different stem cells in a crypt all are ultimately descended from the master stem cell.

Cellular Hierarchy. A cell's position within the hierarchy is usually defined by its ability to function as a stem cell, and physically relates to the cell's position within the crypt.

Cell Turnover. Net cell turnover is defined as the difference between the rate of cell production and the rate of cell loss. Loss may be defined as being from either (1) the proliferative cell population within a tissue, in which case it may be due to either differentiation or cell death; or (2) from the epithelium as a whole, in which case it is due to cell death alone.

Villus. Small projection into the gut lumen of mature, differentiated, epithelial cells. Each villus may be formed from cells derived from up to half a dozen crypts, that is, unlike crypts, they are polyclonal.

Crypt-villus Axis. Specifically refers to the small intestinal epithelium and relates to the direction in which the majority of epithelial cells move during their lifetime as proliferative and, consequently, mature differentiated cells.

## The Intestine

### Cell Production

**The Basics.** Cell turnover in both the small and large intestine is high, with the turnover time for the entire small intestinal epithelium being estimated at approximately 54 hours (in mouse) and for the colon, about 60 hours (mouse) (Cheng and Bjerknes, 1983). Total cell production in the human small intestine has been estimated at being up to $2 \times 10^{11}$ cells per day (Potten, 1995). Cell production takes place at or near the base of the intestinal crypts. Cells then divide several more times as they migrate up the crypt and finally differentiate into mature villus cells with either absorptive, secretory, or endocrine function. A few cells, the Paneth cells, migrate to the base of the crypt and fulfill an antimicrobial role. After a short functional life (3 days), cells are lost from the tips of the villi in the small intestine or from the crypt table in the large intestine (after 5–9 days). Because of its complex morphology, a tight balance needs to be maintained between cell loss and cell gain in the intestinal epithelium in order to maintain its tissue architecture and optimal function: That is, crypt output has to match cell loss from the villi. Primarily, control of cell numbers is exercised at the level of the stem cells, from which all the other intestinal cell lineages are derived. Anotated photomicrographs showing the histological appearance of the small and large intestinal epithelia are shown in Fig. 1.

**Stem Cells.** In the small intestine, the stem cells are thought to be located at about the 4th cell position from the bottom of the crypts, directly above the Paneth cells (Potten et al., 1997). They are suggested to reside in an annulus of 16 cells surrounded by their immediate daughter cells. As yet, there are no defined molecular markers for the stem cells. Their position has been determined on the basis of in vivo DNA labeling experiments that show the origin of cell proliferation and migration to be at the 4th cell position (Qiu et al., 1994). Certain cells at this position also show retention of label, perhaps indicating that they retain a master copy of DNA (Potten et al., 2002). Intriguingly, individual cells with high levels of telomerase expression have also been demonstrated at position 4 (Booth and Potten, 2000), which coincides nicely with the idea of a stem cell with a well-maintained master DNA template that undergoes many rounds of replication during the lifetime of the animal.

Data from studies on chimeric animals (Winton et al., 1988) and on animals demonstrating X-linked enzyme polymorphism (Thomas et al., 1988) suggest that crypts become monoclonal during early (fetal) development and, therefore, all the cells in an individual crypt are ultimately descended from one master stem cell. The precise molecular control of this monoclonal selection process has yet to be defined (see later on in this chapter).

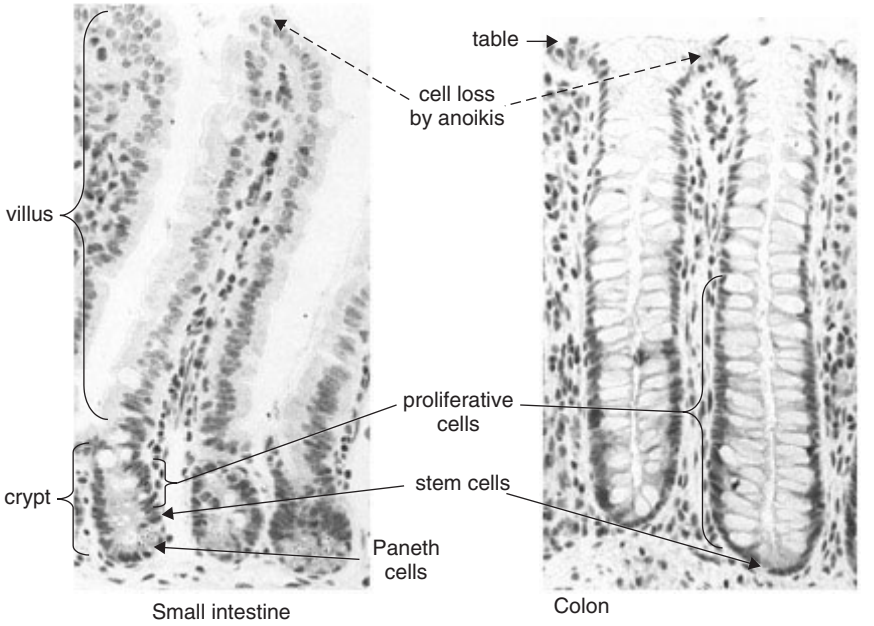


FIGURE 1. The intestinal epithelium.

When a stem cell divides, its usual fate is thought to be forming one stem cell and one daughter cell; the daughter may either divide several more times, migrating up the crypt and differentiating into an absorptive, secretory, or endocrine cell or, alternatively, it may migrate down to the very base of the crypt and differentiate into a Paneth cell. Occasionally, a stem cell division may result in the formation of two equivalent stem cells. There are very few stem cells per crypt (four to six), and so each stem cell's lineage makes a considerable contribution to the crypt's output of 300 cells per day. A single extra stem cell, therefore, can considerably upset the status quo; hence, the need for tight regulation. Consequently, these excess stem cells are deleted by apoptosis, thus preventing crypt hyperplasia and the enhanced possibility of malignant transformation. The scoring of apoptotic events on a cell positional basis reveals a peak in spontaneous apoptosis at the stem cell position near the base of the small intestinal crypts, and this is thought to reflect the deletion of excess stem cells (Potten et al., 1997).

The other way in which crypt cell numbers are regulated is through crypt fission. It has been proposed that crypts bifurcate, thus forming two crypts when crypt/stem cell numbers reach an upper threshold limit. It has been further proposed that there is an ongoing cycle of crypt division, but estimates of crypt cycle times and the number of crypts in cycle vary wildly.

One study suggests that crypts are not in steady state but are constantly expanding until they reach the threshold cell number at which they bifurcate (Totafurno et al., 1987); this cycle time was estimated at 110 days (mouse). Others have proposed that less than one-third of crypts divide during the lifetime of the animal and that crypt cycle time is much greater (>100 months; mouse) (Li et al., 1994).

On some occasions, however, extra stem cells are required to be produced, either to replace single stem cells that have been deleted by apoptosis because they have sustained genomic damage, or to enhance crypt output or allow crypt multiplication through fission, in order to affect epithelial repair following gross mucosal damage. The response of the epithelium to mucosal damage is discussed later.

There is a cellular hierarchy relating to stem cell potential and commitment to differentiated fate in the crypt, based on cell position. Much information on this has been obtained from studies of the response of the epithelium to injury. If all the stem cells are deleted, then there is a cohort of up to six immediate daughter cells that are able to substitute for them and take over as the stem cells and repopulate the epithelium with all cell lineages. If these cells are deleted also, there is a further cohort of between 16 to 24 cells at higher crypt positions that can fulfill this role. Proliferative cells further up the crypt are not able to substitute as stem cells if this latter cohort is deleted, having reached a point of commitment from which there is no turning back (Potten, 1998). Is a cell committed to a particular differentiated fate (absorptive/goblet/endocrine) at this point, or does this occur later when it eventually stops dividing? Some recent studies suggest that long-lived stem cell daughters can only go on to produce cells of one particular lineage. *Dolichos biflorus* agglutinin (DBA) is a lectin that binds to the intestinal epithelial cells of mice that express the genetic locus, *Dlb-1*. Some strains of mice express this locus and some do not. Treating a *Dbl*–/– strain such as Swiss–Webster (SWR) with a mutagen will introduce random genetic mutations into the genome. Some of these may occur with the *Dlb-1* locus and result in its expression, and consequently, epithelial cells may be labeled by the lectin. Following treatment of SWR mice with *N*-ethyl-*N*-nitrosourea, a rapid increase in DBA-positive staining can be observed in the intestinal crypt epithelium (Bjerknes and Cheng, 1999). The majority of these cells disappear within 3 to 4 weeks, but some DBA-positive cells remain for many months. These cells must arise from mutated stem cells or, at least, long-lived progenitor cells. Indeed, such DBA-positive cells can be viewed near the base of the crypts. The interesting thing about these studies is that they revealed that in some crypts the DBA-positive cells were of a single lineage, either columnar (absorptive) or mucus (goblet precursors). Crypts with both lineages were also seen. The initial burst of DBA positivity that disappears must be due to mutations in short-lived progenitor cells. Again, these short-lived cell clones could be of either columnar or mucus lineage, or both.

These results may be interpreted as suggesting that there are both short- and long-lived progenitor cells within crypts that already have a specified fate (columnar or mucus), the latter presumably the immediate daughters of a stem cell. These studies did not suggest from which lineage the endocrine and Paneth cells might be derived. From the data generated, the number of long-lived progenitors (stem cells and immediate daughters) was suggested to be four to five per crypt (Bjerknes and Cheng, 1999). These could correspond to the four to six stem cells identified on the basis of their radiosensitivity.

How is it determined then that stem cells need to be created or deleted by apoptosis and how are stem cells themselves maintained? Also, what determines the ordered proliferation, migration, differentiation, and ultimately the death of epithelial cells? The molecular control of cell turnover in the small intestinal is just beginning to be unraveled. Cell-cell and cell-basement membrane adhesion molecules provide important signals that govern proliferation, differentiation, and apoptosis. Autocrine and paracrine secretory molecules also play a key role in regulating the dynamics of the epithelium. These subjects will now be discussed in more detail.

## Regulation of Epithelial Proliferation, Differentiation, and Apoptosis

### Cell Adhesion Signals

*E-cadherin.* The cell-cell adhesion molecule, E-cadherin, has been shown to regulate cell proliferation, migration, and apoptosis in the small intestinal (SI) epithelium in a chimeric mouse model (Hermiston et al., 1996; Hermiston and Gordon, 1995). Disruption of E-cadherin function through the overexpression of a dominant negative N-cadherin in SI epithelium results in increased apoptosis and proliferation, loss of cell polarity and perturbed cell migration along the entire crypt–villus axis, and apparent pathology (neoplasia and inflammatory bowel disease; Hermiston and Gordon, 1995). In contrast, forced expression of wild-type E-cadherin results in reduced numbers of crypt mitoses and perturbed patterns of epithelial cell apoptosis (Herminston et al., 1996). The effects of cadherins on proliferation/migration/apoptosis are mediated through their interaction with Armadillo-family proteins such as β-catenin, plakoglobulin, and desmoplakin, which link cadherin adhesion junctions with cytoskeletal elements (Kikuchi, 2000; Nagafuchi, 2001; Jamora and Fuchs, 2002). Also, E-cadherin may regulate the free cytoplasmic levels of β-catenin, which in its monomeric form can act as a transcriptional regulator to promote cell proliferation.

*Laminins.* Signal transduction from extracellular matrix (ECM) components (e.g., laminins) through their cell surface receptors, integrins, is also

crucial in providing signals controlling proliferation, differentiation, and apoptosis. Laminins are multisubunit glycoproteins, consisting of α, β, and γ chains. Both laminins and integrins show differential distribution along the crypt–villus axis. In human SI epithelium, expression of laminin-2 appears to be specifically restricted to the base of the crypts, whereas laminin-1 and -5 show villus-specific expression (Bouatrouss et al., 2000). This pattern of expression may reflect their possible role in defining/maintaining the function of the enterocytes found in these specific regions. Laminin-1 expression is associated with the expression of differentiation markers in vitro, such as sucrase isomaltase, and presumably has the same role in promoting differentiation of villus enterocytes in vivo. This relationship may be mediated via laminin-1-regulated expression of the Caudal-related transcription factor, Cd×2 (Lorentz et al., 1997), possibly signaled through α7Bβ1 integrin (Basora et al., 1997).

*Integrins.* Integrins consist of two subunits, α and β, that determine their ligand specificity and their cytoplasmic interactions. Two integrins, α3β1 and α6β4, have been implicated in the migration of enterocytes along the crypt–villus axis, primarily through data acquired from models of intestinal epithelial would healing (Mecurio et al., 2001). The β4 subunit is particularly interesting; its cytoplasmic domain is different from other β subunits and it is able to self-associate, meaning that it functions in signaling pathways independently of laminin binding by its associated α chain (Rezinczek et al., 1998). Mice with defective β4 demonstrate impaired crypt epithelial cell proliferation and this is associated with increased expression of the CDK inhibitor, $p27^{Kip1}$ (Murgia et al., 1998).

In addition to cell migration within the intestinal epithelium, in vitro studies on isolated colonic crypts demonstrate that the β1 subunit is responsible for transducing antiapoptotic signals, following the engagement of matrix components such as collagen 1 (Sträter et al., 1996). Constitutive overexpression of integrin α5β1 in intestinal epithelial cells in vitro also protects against a range of apoptotic stimuli (Lee and Juliano, 2000). However, the in vivo role of α5β1 and its interaction with its major ligand, fibronectin, in controlling enterocyte survival are not yet defined.

*Laminin/Integrin Signaling.* The binding of laminins by integrins results in integrin clustering and the recruitment of a number of different cytoplasmic proteins, including kinases and structural proteins, to form complexes called focal adhesions (Miranti and Brugge, 2002; Schwartz, 2001). One of the key proteins in these foci is focal adhesion kinase (pp125FAK; for a review, see Schaller, 2001), which undergoes autophosphorylation on defined tyrosine residues in response to integrin ligation. Phosphorylated pp125FAK can, in turn, recruit and phosphorylate components of the Ras/MAP kinase signaling pathway, such as Grb2/SOS, to ultimately

initiate Elk-1-mediated transcriptional events such as upregulated expression of cyclin D1. Alternatively, it can recruit and activate PI-3 kinase, the consequence of which is activation of AKT/PK-B that, in turn, phosphorylates and inactivates (via sequestration) the pro-apoptotic Bcl-2 family protein, Bad. It has been demonstrated recently that pp125FAK undergoes specific cleavage by caspase-3 and -6 during the apoptosis of isolated, human colonic epithelial cells in vitro (Grossmann et al., 2001).

Integrins may also serve to regulate signaling via growth factor receptors (GFRs) (reviewed by Yamada and Even-Ram, 2002). This interaction may result from coclustering of integrins and GFRs and mutually enhanced ligand binding (Miyamoto et al., 1996). Alternatively, integrin ligation may result in the phosphorylation of the cytoplasmic, signaling domain of the GFR, independent of GFR-ligand binding (Moro et al., 2002). Specifically, ligation of $\alpha_v\beta_3$ integrin or fibronectin binding to $\beta_1$-containing integrins can induce activation of epidermal growth factor receptor (EGFR), dependent on the protein tyrosine kinase, c-Src, and the adaptor protein, p130Cas (Moro et al., 2002). This effect is independent of EGF binding to its receptor and interestingly, independent of pp125FAK. This mechanism could contribute to the antiapoptotic signaling of $\beta_1$ integrins discussed previously.

These results show that cell–cell contacts and cell–ECM interactions are important in providing survival and proliferative signals to epithelial cells. Apoptosis that results from the loss of signals provided by adhesion molecules is given the more specific name of anoikis (Frisch and Francis, 1994). Excellent reviews on cell adhesion and apoptosis (Gilmore and Streuli, 1998) and anoikis mechanisms (Frisch and Screaton, 2001) are available. The loss of cells at the villus tip is also probably due to anoikis. Electron microscopic studies clearly demonstrate the morphological changes occurring in cells being lost at the villus tip, in particular the loss of microvilli (Potten and Allen, 1977). Labeling of DNA strand breaks also shows positive cells at the tips of the villi, consistent with the cells undergoing apoptosis (Hall et al., 1994; Shibahara et al., 1995).

The increased expression of J1/tenascin, which increases repulsion between epithelial cells and basement membrane, is also observed in murine intestinal villi and may contribute to the loss of cells from the villi by anoikis (Probstmeier et al., 1990). An elegant study by Rosenblatt et al. (2001) demonstrates the precise changes occurring in dying cells in simple epithelium in vitro, mechanisms that probably apply also to cell loss in the intestine. These studies show that apoptotic cells are extruded from epithelial layers by an active process which involves the contraction of neighboring cells, by an actin/myosin-dependent process, so they close in underneath the apoptotic cell and squeeze it out of the monolayer. This process is aided by the general contraction of the apoptotic cell itself. In this way, the integrity of the monolayer and hence barrier function can be maintained despite the loss of individual cells.

## Paracrine and Autocrine Signaling

*Wnt Signaling.* Wnt proteins are secretory glycoproteins that play an important part in directing cell fate during development. More than a dozen Wnts have been identified, many with different isoforms, and they show a high level of conservation across different species, emphasizing their basic importance in cell regulation. Roel Nusse at Stanford University maintains an excellent site on Wnts at ⟨http://www.stanford.edu/~rnusse/wntwindow.html.⟩ Wnts signal through the cell surface receptors of the Frizzled family. Multiple Frizzled members across various species have been cloned and show ligand specificity. Wnt/Frizzled interaction may either affect β-catenin-regulated transcription, or $Ca^{2+}$-dependent signaling (Miller et al., 1999; Kühl et al., 2000), depending on the subtype of Frizzled receptor.

*Wnt Ligands and the Intestinal Epithelium.* Most studies on the role of this signaling pathway in regulating intestinal epithelial cell fate, during development and in adult tissue, primarily focus on the downstream components of the pathway, such as β-catenin. Studies on the expression Wnt ligands in the mammalian intestine are limited to studies of gene expression during gut development in the mouse (Lickert et al., 2001) and the dysregulated expression of *Wnt*s in colonic tumors (Dimitriadis et al., 2001; Katoh et al., 1996; Vider et al., 1996). *Wnt*s *4* and *13* are expressed in the embryonic and adult small intestine, respectively (Lickert et al., 2001; Katoh et al., 1996); *Wnt 11* is expressed in embryonic colon (Lickert et al., 2001); *Wnt*s *2*, *5a*, *11*, and *13* are expressed in the adult colon (Dimitriadis et al., 2001; Katoh et al., 1996; Vider et al., 1996); and *Wnt*s *2*, *4*, *5a*, *6*, and *7a* are reported to be overexpressed in colonic tumors (Dimitriadis et al., 2001; Vider et al., 1996).

What controls the expression of the *Wnt* genes themselves is not well characterized, although specific *Wnt*s have been shown to be regulated by Sonic hedgehog (see section that follows; Reddy et al., 2001) and by ECM components such as collagen (Bui et al., 1997).

*β-Catenin-Regulated Transcription.* Free (monomeric) β-catenin is transcriptionally active via its heterodimerization with T cell factor 4 (Tcf-4), and other members of the high mobility group (HMG) of transcription factors. The levels of free β-catenin are tightly controlled through its targeted degradation by the ubquitination pathway (Miller and Moon, 1996; Kikuchi, 2000). In the absence of Wnt ligands, free β-catenin is constantly degraded, a process directed by the proteins Axin, APC (the product of the *Apc* gene, mutated in familial andenomatous polyposis coli; Nishisho et al., 1991) and glycogen synthase kinase-3β (GSK-3β). There is some experimental evidence to suggest that E-cadherin expression can also regulate the level of monomeric β-catenin within the cell (Fagotto et al., 1996; Heasman et al.,

1994). The transcriptional activity of β-catenin is also regulated by other mechanisms, which will be described in a subsequent section. Binding of Wnt ligands to their cell surface receptor Frizzled results in the G-protein-dependent activation of Dvl (Deshevelled), which in turn inhibits the action of GSK-3β and prevents β-catenin degradation, allowing its association with HMG transcription factors.

Experimental evidence from a *Tcf-4−/−* mouse model suggests that Tcf-4/β-catenin signaling is essential for the maintenance of epithelial stem cells in the small intestine during development (Korinek et al., 1998). Further evidence of the importance of β-catenin signaling for intestinal stem cells is provided by studies using a chimera constructed by injecting embryonic stem cells (derived from a sv129 mouse), expressing a lymphocyte enhancer factor-1 (Lef-1)/β-catenin fusion protein, into the blastocysts of B6-ROSA26 transgenic mouse (Wong et al., 2002). Lef-1 is another HMG transcription factor. ROSA26-derived crypts express β-galactosidase and can be stained blue following tissue isolation and incubation with X-gal: sv129 epithelium remains unstained. The expression of the transgene results in increased apoptosis in the sv129 intervillus epithelium (which ultimately forms the crypts in the developing gut), in the absence of the increased proliferation: This effect is observed 2 days prior and 1 day after birth—at this time the crypts are still polyclonal. ROSA26 crypts that do not express the fusion protein show no such response. Two weeks after birth, only ROSA26-derived crypts remain. In chimaeras that do not express the transgene, both monoclonal ROSA26 and sv129 crypts (with polyclonal villi) can be observed; therefore, expression of the Lef-1/β-catenin transgene must result in the deletion of stem cells as the intestinal epithelium develops, probably through the induction of apoptosis.

These studies suggest that the absolute level of β-catenin signaling determines stem cell fate (Wong et al., 2002), there being a minimum signaling requirement for stem cell survival (Korinek et al., 1998) and a threshold beyond which stem cell fate is apoptosis (Wong et al., 2002). This mechanism has been proposed to explain the tight regulation of stem cell numbers within the crypts and the formation of monoclonal crypts during development (Wong et al., 2002; see Fig. 2). It is possible that the response may have intermediate levels between these two extremes, with there being optimal levels of β-catenin signaling that support proliferation and differentiation. There are data suggesting that downregulation of β-catenin signaling in proliferative enterocytes is required to allow differentiation (Mariadason et al., 2001b).

In the adult intestine, the expression of Tcf-4 is highest in cells further along the crypt–villus axis, up and away from the stem cell compartment (Barker et al., 1999). Cells in the upper crypt and villus are more refractory to apoptosis induction than the stem cells at the base (Potten, 1990; Wilson et al., 1998); therefore, Tcf-4/β-catenin signaling in these cells cannot be at a

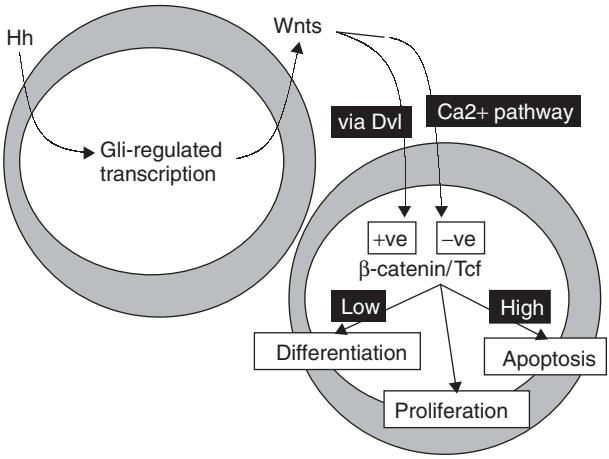


FIGURE 2. Proposed role of β-catenin/Tcf signaling in directing cell fate.

sufficient level to induce apoptosis, although the cells of the upper crypt and villus are committed to their fate and will be dead within 3 days.

The promotion of cell survival and proliferation by Tcf-4/β-catenin (Korenik et al., 1998; Chen et al., 2001) are mediated via regulating the expression of a number of genes including those coding for cyclin D1 (Qiao et al., 2001) and the transcriptions factors Cdx-1 (Lickert et al., 2000) and c-myc (He et al., 1998). Cdx-1 demonstrates an almost identical expression pattern to Tcf-4, being expressed in the proliferative cell compartment, above the stem cells and Paneth cells, in the SI crypts (Subramanian et al., 1998). The effect of Cdx-1 on proliferation is associated with its ability to upregulate the expression of the C-lectin domain-containing, pancreatitis-associated protein 1 (PAP1) (Moucadel et al., 2001). The mode of action of PAP-1 (which is a secretory protein) is not clear, although C-type lectins are known to bind integrins (Matsumoto et al., 2001; Marcinkiewicz et al., 2000).

The related transcription factor, Cdx-2, shows a contrasting pattern of expression being expressed just in the villi (that is, in differentiated cells; James and Kazenwadel, 1991) and is associated with the expression of differentiation markers, such as lactase (Fang et al., 2000) and the vitamin D receptor (Yamamoto et al., 1999).

*The Wnt/$Ca^{2+}$ pathway*. In addition to their ability to regulate Disheveled, Wnts can also stimulate phospholipase C in a G protein-dependent manner, via ligation of Frizzled-2, to liberate inositol 1,4,5-triphosphate ($IP_3$) and diacylglycerol (DAG), with the consequent activation of $Ca^{2+}$-Calmodulin-dependent kinase II (CamKII) and protein kinase C (Malbon et al., 2001). Recently, the transcription factor, nuclear factor of activated T cells (NF-AT),

was identified as a downstream target of the Wnt/$Ca^{2+}$ pathway in *Xenopus* (Saneyoshi et al., 2002). Also, Wnt/$Ca^{2+}$ signaling can inhibit Wnt/β-catenin signaling. Again in *Xenopus*, the Wnt-dependent activation of PKC results in phosphorylation and inactivation of Dishevelled and CamKII functions to phosphorylate specific β-catenin-binding partners, such as the HMG transcription factor LEF, in order to prevent their homodimerization with β-catenin (Kühl et al., 2001).

Of the *Wnt* genes identified as being expressed in the GI tract (*Wnts* 2, *4*, *5a*, *6*, *7a*, *11*, and *13*; see the previous discussion), the Wnt proteins 5a and possibly 4 and 11 function to activate the $Ca^{2+}$ signaling pathway (Kühl et al., 2000).

*Hedgehog Signaling.* *hedgehog* is a *Drosophila* gene coding for a secretory growth factor involved in establishing segmental polarity (for an extensive review, see Ingham and McMahon, 2001). Three mammalian homologues of *hedgehog* have been identified. They are Sonic *hedgehog (Shh)*, *Indian hedgehog (Ihh)*, and *Desert hedgehog (Dhh)*. Hedgehogs are synthesized as precursor proteins (c. 45 kDa size) that undergo autocatalytic processing to yield an N terminal signaling polypeptide (c. 20 kDa), which interacts with two transmembrane proteins, Patched (Pct) and Smoothened (Smo). Ligation of Pct derepresses the activity of Smo and results in Smo-mediated activation of the Gli family of transcription factors, which regulate the expression of a range of genes including *Wnts* (Reddy et al., 2001; Mullor et al., 2001) and *Bone morphogenic protein 4* (*Bmp-4*; Roberts et al., 1995).

During gut development, both S*hh* and *Ihh* are expressed in the intervillus epithelium, which is the location of the stem cells and ultimately gives rise to the crypts (Ramalho-Santos et al., 2000). This expression pattern is the same as that for Tcf-4 (Korinek et al., 1998). Genes coding downstream components of the signaling pathway, such as *Ptc* and *Gli* and *Bmp-4*, are expressed in the subepithelial mesenchymal cells, indicating the importance of this signaling pathway in epithelial–mesenchymal cross-talk. Like Tcf-4, Ihh appears to play an important role in the maintenance of the stem cell population during intestinal development. On day 18.5 of fetal development, *Ihh*−/− mice show greatly reduced numbers of proliferating cells within the intercrypt region, a reduction in mature villus enteroendocrine cells, and villus atrophy (Ramalho-Santos et al., 2000). The effect of the lack of Ihh signaling is, however, independent of any change in Tcf-4 expression. It is not clear what the downstream targets of Ihh signaling in the intervillus/crypt cells are that maintain the stem cell population; however, Ihh-dependent expression of the transcription factor Pdx-1 appears to be essential for maintenance of the enteroendocrine lineage. Pdx-1 expression is absent from the villi of *Ihh*−/− mice (Ramalho-Santos et al., 2000); previously, *Pdx-1*−/− mice have also been found to lack cells of the enteroendocrine lineage (Offield et al., 1996).

*Forkhead Transcription Factors.* These are a diverse range of transcription factors that share a conserved winged helix DNA binding domain. Initially described in *Drosophila*, a wide range of mammalian homologues have now been described that include hepatocyte nuclear factors (HNFs), forkhead homologues (FHHs), human forkheads (HFFs), and murine forkheads. Forkhead signals are known to be important in regulating cell cycle progression and apoptosis. The forkheads AFX and FKHR-L1 upregulate Rb-like p130 protein to induce G0 (quiescence; Kops et al., 2002); FKHR upregulates the cyclin-dependent kinase inhibitor $p27^{kip1}$ to induce arrest in G1 (Nakamura et al., 2000); also, FKHR-L1 upregulates the expression of Gadd45a to allow cycle arrest and DNA repair during G2/M (Tran et al., 2002). In addition, forkheads have been shown to drive apoptosis. AFX regulates the expression of the transcriptional repressor BCL-6, which in turn downregulates the expression of the key antiapoptotic protein, Bcl-$X_L$, by binding directly to the *Bcl-$X_L$* promoter (Tang et al., 2002). FKHR-L1 is known to upregulate the pro-apoptotic Bcl-2 protein Bim (Dijkers et al., 2000).

Forkheads are downstream targets for many cell survival-signaling molecules including insulin and insulin-like growth factor 1 (IGF-1; Nakae et al., 2000), Il-2 (Stahl et al., 2002), and TGF-β (Shin et al., 2001). These survival ligands are known to activate the serine/threonine kinase, Akt/PKB, resulting in the phosphorylation of forkheads and their subsequent sequestration by the cytoplasmic protein 14-3-3, thus repressing forkhead activity and apoptosis and promoting cell proliferation (Brunet et al., 1999; Shin et al., 2001; Dijkers et al., 2002; Kops et al., 2002).

In the intestine, forkheads are found in both epithelial and mesenchymal cells. Forkhead genes expressed in the epithelium include *HFH11*, *HNF3-β*, and *Fkh6* (Ye et al., 1997; Kaestner et al., 1997). Expression of *HFH11* and *HNF6* is specific to the proliferative compartment of the epithelium (there is some mesenchymal expression also). *Fkh6* is only expressed in the subepithelial mesenchymal cells; however, gene knockout models suggest that it plays an important part in regulating epithelial cell proliferation. *Fkh6*−/− mice show deregulated crypt cell proliferation and abnormal crypt morphology, with a general increase in the epithelial cell numbers and a specific increase in the number of goblet cells (Kaestner et al., 1997). These results emphasize the role of forkheads in controling cell numbers through the repression of proliferation and promotion of apoptosis. Also, they demonstrate the importance of epithelial–mesenchymal communication.

*Notch Signaling.* Notch is a cell surface receptor that is sequentially cleaved by a series of proteases upon binding of ligands such as Jagged and Delta (Weinmaster, 2000). Proteolysis releases the intracellular domain of Notch (NICD), which translocates to the nucleus where it regulates transcription in combination with the DNA-binding protein CSL (Jarriault et al., 1995). One of the genes regulated by Notch is *Hes-1*, which codes for a basic

Helix-Loop-Helix (bHLH) transcriptional repressor (Jarriault et al., 1998). Hes-1 is important in the intestinal epithelium. Results from *Hes-1−/−* mice suggest that Hes-1 suppresses the specification of endocrine cell fate for villus epithelial cells during embryogenesis, as *Hes-1−/−* mice have greatly increased numbers of mature villus endocrine cells on day E17, just prior to birth (Jensen et al., 2000). Hes-1 is normally expressed by villus enterocytes during embryogenesis, but not by the cells of the intercrypt epithelium. In adult mice, Hes-1 *is* expressed in the proliferating cells of the crypt but *not* in the villus, suggesting that its expression is downregulated to allow differentiation. Other bHLH transcriptional repressors such as the Ids also inhibit differentiation (hence, their name Id) and promote proliferation. Ids are also expressed in the intestinal epithelium and their dysregulation may be important in colorectal cancer (see discussion that follows).

*TGF-β Signaling.* The polypeptide growth factor TGF-β plays an important role in suppressing cell proliferation and promoting cellular differentiation in epithelial tissues (Yue and Mulder, 2001). Inhibition of cell proliferation is achieved through targeted expression of the CDK inhibitors such as $p15^{INK4b}$ and $p21^{WAF\text{-}1/Cip1}$ and repression of c-myc. All three isoforms of TGF-β are present in the adult intestinal epithelium. Recent studies show that TGF-$\beta_2$ is specifically expressed and presumably secreted by entro-endocrine cells and TGF-$\beta_3$ by goblet cells (Dünker et al., 2002).

Both *TGF-$\beta_2$+/−* and *TGF-$\beta_3$+/−* mice show significant increases in villus length and significant decreases in intestinal epithelial cell apoptosis at the villus tips, in association with increased Bcl-2 and Bcl-$X_L$ expression (Dünker et al., 2002). The inference from these results is that TGF-β isoforms secreted by mature enterocytes regulate the death of cells as they reach the villus tip, which coincidentally show the highest expression of the Type II TGF-β receptor (TGF-β-RII), although it is expressed along the length of the crypt–villus axis.

TGF-β-RII expression is positively regulated by the ETS transcription factor E74-like factor 3 (Elf3). *Elf3−/−* mice show gross developmental abnormalities of the intestine (Ng et al., 2002). Villus development is delayed during embryogenesis and villi are sparse and abnormal in neonatal animals. The absorptive, columnar cells also have an unusual appearance and lack of microvilli, and there is a significant reduction in mature goblet cells. These effects are associated with a decreased expression of TGF-β-RII by the epithelial cells, but not by lamina propria cells, again highlighting the importance of TGF-β signaling for the intestinal epithelium.

The expression of TGF-β-RII is also regulated by the Kruppel-like transcription factors Sp1 (positively, Liu et al., 2000) and Sp3 (negatively, Ammanamanchi and Brattain, 2001) in MCF-7 breast adenocarcinoma cells. Sp1 and Sp3 are known to be expressed in intestinal epithelial cell lines (Aslam et al., 2001; Gartel et al., 2000), and Sp1 positively regulates TGF-β-

RII in colon carcinoma cells also (Periyasamy et al., 2000). Other experiments show epithelial differentiation is associated with a high Sp1:Sp3 ratio and, consequently, support the hypothesis that TGF-β-RII expression is important in the differentiation of epithelial tissues.

Sp1 and Sp3 regulate the production of Type 2 Mucin (MUC2) by intestinal epithelial cells. The *MUC2* promotor contains GC-rich boxes that act as Sp1/Sp3 binding sites (Aslam et al., 2001), and *MUC2* transcription is dependent on the methylation status of the promotor, with increased methylation resulting in reduced expression (Gratchev et al., 2001). It is known that Sp1 acts synergistically with other transcription factors, including Kruppel-like factor-4 (Klf-4), to regulate the transcription during differentiation (Higaki et al., 2002). Mice null for Klf-4 expression have abnormal goblet cells, reduced goblet cell numbers (10% of wild-type), and altered MUC2 expression (Katz et al., 2002). Mice null for MUC2 expression show a more extreme phenotype with increased epithelial cell proliferation and migration, decreased apoptosis, and elongated crypts (Velcich et al., 2002). Precisely why the absence of MUC2 has these effects is not known.

*Glp-2.* Glucagonlike peptide-2 (Glp-2) is another secreted polypeptide growth factor that has a positive influence on enterocyte proliferation. It is produced by a subset of enteroendocrine cells (L cells), it is thought in response to nutrient signals from the gut lumen. It acts to promote specifically the proliferation of columnar (absorptive) epithelial cells in the SI, and this is associated with increased c-Fos expression in these cells. However, the effect of Glp-2 on the columnar cells is indirect. Glp-2 receptors are only found on enteric neurons and not epithelial cells, and it appears that a second proliferative signal is secreted by the enteric neurons in response to the Glp-2 secreted by the L cells (Bjerkes and Cheng, 2001).

Bcl-2 Family Proteins. In the colon, tissue architecture is simpler than in the SI. There are longer crypts but no villi, with the crypts opening out on to an intercrypt, table region. Stem cells are located at the base of the colonic crypts; again evidence for this comes from DNA labeling studies showing the 1st cell position to be the origin of cell proliferation and migration (Potten, 1995). Studies indicate that there are fewer stem cells in large bowel crypts compared to the small bowel, perhaps just one, and this correlates well with reduced cell turnover and increased transit times for epithelial cells along the length of the crypts. There is less spontaneous apoptosis in the large bowel (Potten et al., 1997). This is partly explained by there being fewer stem cells, but also spontaneous apoptosis in colonic stem cells is suppressed by their expression of Bcl-2 (Merritt et al., 1995). This role for Bcl-2 is highlighted by studies using *bcl-2−/−* mice, which show significantly elevated levels of spontaneous apoptosis at the base of their colonic crypts but not in their small intestinal crypts (Merritt et al., 1995). Colonic epithelium from

*bcl-2*–/– mice also shows elevated expression of the pro-apoptotic, Bcl-2 family protein, Bad (Wilson and Potten, 1996). The consequences of Bcl-2 expression by colonic stem cells are later discussed below.

## Response of the Intestinal Epithelium to Injury

This is an important area of study as gastrointestinal toxicity is a major limiting factor in systemic radio- and chemotherapy for cancer. Studies of radiation and cytotoxic drug-induced injury have yielded important information regarding the hierarchical cellular organization in the crypts (Potten and Grant, 1998). The epithelia of the small and large intestine respond very differently to cell injury. Reflecting its greater cell turnover and higher rates of spontaneous apoptosis in adult, normal tissue, the small intestine shows higher levels of apoptosis in response to injury from exposure to ionizing radiation (IR) or chemotherapeutic agents (Potten et al., 1992; Merritt et al., 1995; Wilson et al., 1998). The proliferative cells of the crypts, particularly those within the putative stem cell zone, show much greater sensitivity than the differentiated villus cells (Wilson et al., 1998). The exquisite sensitivity of cells at the base of the SI crypts to apoptotic stimuli has been recognized for many years and well characterized in a number of studies (Potten, 1977, 1990). It is only more recently that the molecular basis for the differences in apoptotic sensitivity between the SI and the colon has been established, primarily through the use of knockout and transgenic mouse models (Watson and Pritchard, 2000).

Regulators of Apoptotic Response—Bcl-2 Family Proteins and p53. As with levels of spontaneous apoptosis, the sensitivity of enterocytes to apoptotic stimuli such as ionizing radiation is dependent on Bcl-2 expression. Colonic crypts from bcl-2 null mice show elevated levels of IR-induced apoptosis compared to wild-type animals (Merritt et al., 1995). *Bcl-2* status is not important for SI response, but another *bcl-2* family member, *bcl-w*, is, with *bcl-w*–/– mice showing increased SI apoptosis following administration of 5-fluorouracil (Pritchard et al., 2000).

Other determinants of major importance in the dichotomy of sensitivity between the SI and the colon are p53 and the p53-regulated gene product, the CDK inhibitor, $p21^{WAF\text{-}1/cip1}$. The acute apoptotic response (within 4–6 hours), to IR in the intestinal epithelium, is entirely dependent on p53 in both the colon and SI (Merritt et al., 1994, 1997; Clarke et al., 1994); however, both p53 and $p21^{waf\text{-}1/cip1}$ are differentially expressed in the SI and colonic crypts following IR exposure (Wilson et al., 1998). In the SI crypts, the peak frequency of p53 expression at 3 to 4 hours (postirradiation) is twice that observed in colonic crypts (Merritt et al., 1994; Wilson et al., 1998). In the colonic crypts, although the frequency of p53 expression is less, it is maintained over a longer time period (up to 96 hours), and this is associated with

an accumulation of $p21^{WAF\text{-}1/cip1}$-positive cells in the colonic crypts to a very high level (almost 40% of all crypt cells by 72 hours) and associated cell cycle arrest. $p21^{WAF\text{-}1/cip1}$ expression is lower in the SI crypts and cell cycle arrest is quickly overcome (within 24 hours) (Wilson et al., 1998).

It might be argued from these studies that $p21^{WAF\text{-}1/cip1}$ expression plays an important role in protecting cells (i.e., in the colon crypts and at the top of SI crypts) against apoptosis by promoting cell cycle arrest. Studies on *p21*$^{WAF\text{-}1/cip1}$*–/–* cell lines, grown in vitro and as xenografts, show that $p21^{WAF\text{-}1/cip1}$ deficiency prevents cell cycle arrest *and* increases the sensitivity of the cells to radio/chemotherapeutic agents (Waldman et al., 1996, 1997). These observations can be allied with the fact that SI villus cells do not cycle and are not responsive to IR. Absence from the cell cycle or cell cycle arrest do not, however, appear to be dominant influences in determining apoptotic response. Restoration of villus cells into cycle by forced expression of SV40 large T antigen does not confer apoptotic sensitivity to IR (Coppersmith and Gordon, 1997). Data from several studies suggest that it is the absolute level of p53 expression that a cell is able to produce in response to a DNA damage event that determines its fate (Chen et al., 1996; Ronen et al., 1996; Lassus et al., 1996). In the studies on intestinal epithelium, the frequency distribution of cells showing the strongest immunoreactivity of p53 correlates well with the frequency distribution for apoptosis in SI crypts, and the frequency distribution of the remainder of the p53-positive cells (those with weak/moderate p53 immunoreactivity) correlates well with the frequency distribution of the $p21^{WAF\text{-}1/cip1}$-positive cells, at least during the acute phase of the response (Merritt et al., 1994; Wilson et al., 1998).

It should be noted that p53-independent apoptosis, subsequent to arrest in G2/M, does occur in *p53–/–* mice, although with a longer time course (after 12–24 hours) than p53-dependent apoptosis (Clarke et al., 1997; Merritt et al., 1997). Also, spontaneous apoptosis, that is, homeostatic regulation of stem cell numbers is p53-independent: Its positional frequency is unaltered in *p53–/–* mice (Merritt et al., 1994).

The pro-apoptotic, Bcl-2 family protein Bax is another p53-regulated gene product (Miyashita et al., 1994). Bax expression, like $p21^{WAF\text{-}1/cip1}$, is upregulated in IECs following IR exposure (Kitada et al., 1996). However, *bax–/–* mice show normal levels of apoptosis in response to IR (Pritchard et al., 1999). This may reflect functional redundancy within the pro-apoptotic Bcl-2 proteins expressed in the IECs. Another pro-apoptotic, Bcl-2 family protein Bak (Moss et al., 1996) is also upregulated following apoptotic stimuli.

Cytokines. Local cytokine signaling plays an important role in determining the sensitivity of IECs to apoptosis. The cytokine IL-7 and its receptor are expressed by several cell types, including IECs. IL-7Rα*–/–* mice exhibit impaired T and B cell development, and have few mature peripheral or mucosal lymphocytes and no Peyer's patches. These mice have no appar-

ent defect in IEC apoptosis under normal conditions. However, they show enhanced IEC apoptosis in response to IR compared to wild-type controls, which is associated with sustained, elevated levels of Bak (Welniak et al., 2001). The lack of mucosal lymphoid cells does not seem to be the reason for the sensitization, as other lympho-deficient mice such as Rag1−/− do not show such a response. Il-7 appears, therefore, to be specifically involved in promoting IEC survival following cell injury. IL-11 is another cytokine that can regulate IEC survival following IR exposure or administration of chemotherapeutic agents in animal models (Du et al., 1994), through the suppression of apoptosis and the stimulation of cell proliferation within SI crypts (Orazi et al., 1996). Endogenous Il-11 expression is also upregulated in the intestinal mucosa following luminal exposure to alcohol (Fleming et al., 2001). As yet, it is not clear whether IL-11 exerts its pro-survival effects on epithelial cells directly or indirectly, via mesenchymal signals.

Vascular Endothelial Cells. Nonepithelial cell types are important in regulating crypt epithelial survival after radiation-induced injury. Mucosal vascular endothelial cell (MCECs) undergo ceramide-dependent apoptosis in response to lethal doses of whole-body, γ-radiation (doses of 12–15 Gy; Paris et al., 2001), which can be attenuated by administration of bFGF. The consequence of this is delayed mortality in animals exposed to 15 Gy radiation and prolonged survival of irradiated animals that have also received autologous marrow graft. Maintenance of the microvasculature presumably aids the regeneration of the crypt units from surviving stem cell daughters, as evidenced by improved mucosal histology after radiation, through maintaining supply of nutrients and oxygen to the epithelium. MVEC apoptosis is unlikely to contribute to epithelial cell apoptosis in response to sublethal doses of ionizing radiation.

## Apoptosis, Barrier Function, and Inflammatory Disease

As discussed above, epithelial tissue is able to tolerate the loss of cells via apoptosis without compromising its barrier function (Rosenblatt et al., 2001). Even when substantial apoptosis is induced, barrier function (as measured by transepithelial resistance) can be maintained in the short term (<6 hours). Studies in colorectal adenocarcinoma cell line T84 suggest that barrier function is lost, as a consequence of apoptosis induction, if cell monolayers are studied over a longer time period (>24 hours; Abreu et al., 2001). However, permeability increases are dependent on the apoptotic stimulus. Permeability changes are limited in Fas-induced apoptosis to small molecules (<3 kDa), although UV-induced apoptosis results in more profound changes to larger molecules.

Damage to the epithelium, ulceration, and consequent impairment of epithelial barrier function occur in inflammatory bowel diseases, such as

Crohn's (Secondulfo et al., 2001; Irvine and Marshall, 2000) and ulcerative colitis (Nejdfors et al., 1998; Den Hond et al., 1998). Similar changes are also observed in animal models of colitis (Venkatraman et al., 2000; Kitajima et al., 1999). Multiple mechanisms probably contribute to loss of epithelial cells, including death-receptor-induced apoptosis, in response to pro-inflammatory cytokines such as TNF-α and IFN-γ, and anoikis, in response to matrix metalloproteinase-mediated degradation of extracellular matrix components such as laminins.

Following damage to the gastrointestinal epithelium, either by inflammatory processes (i.e., Crohn's disease) or cytotoxic agents (i.e., following cancer chemotherapy), repair processes will be initiated. Key to this repair process is the trefoil protein (so-called due to its tertiary structure) trefoil factor 3 (TFF3, also known as the intestinal trefoil factor), which is primarily expressed by goblet cells (Chinery et al., 1992; Mashimo et al., 1995). This protein and other trefoil proteins such as TFF1 (aka pS2) and TFF2 (aka SP) are upregulated in response to inflammation-induced injury (Wright et al., 1993; Podolsky et al., 1993; Alison et al., 1995) and hypoxia (Furuta et al., 2001). They are highly resistant to degradation, presumably a requisite for their functionality in a protease-rich inflammatory site, and appear to stimulate the migration of epithelial cells into the area of damaged mucosa.

Mice that lack TFF3 protein show reduced ability to repair GI mucosal damage and high mortality following treatment with dextran sulphate sodium, which usually causes just mild colitis in wild-type mice (Mashimo et al., 1996). These studies also demonstrated that exogenous TFF3 could enhance reepithelialization and improve healing following acetic acid-induced damage to the gastric mucosa. Forced overexpression of growth hormone (GH) can promote intestinal epithelial regeneration and upregulate *TFF3* expression (Williams et al., 2001); however, the physiological role of GH in GI mucosal repair is not known. The precise mechanism of TFF3 function is not clear, although it can regulate MAP kinase signaling via inhibition of ERK phosphorylation (Kanai et al., 1998).

There is evidence to suggest that the epithelial cells which migrate into areas of mucosal damage constitute a distinct cell lineage, termed the ulcer-associated cell lineage (UACL; Wright, 1998). These cells arise from the stem cells in crypts adjacent to areas of damage, in response to undefined stimuli resulting from the ulceration. The buds of UACL cells from these crypts then merge together to form a tubular, glandular network not dissimilar from the Brunner's gland in the duodenum (Ahnen et al., 1994). A duct from this glandular structure then arises that emerges onto the mucosal surface, from which dividing UACL cells in the neck of the duct repopulate the epithelium. UACL cells express all three trefoil factors; in addition, they express the epidermal growth factor (EGF), lysozyme and have an altered pattern of mucin expression compared to other enterocytes (Ahnen et al., 1994). These cells may also upregulate TFF1 expression in neighboring, normal epithelium.

## Dysregulation of Enterocyte Survival and Proliferation in Intestinal Cancer

It is well characterized that the suppression of apoptosis and dysregulated proliferation are specifically associated with neoplastic conditions. With regard to the intestine, the development of colon cancer is very common in adult humans. Over 30% of all cancer deaths in Western Europe and North America can be attributed to colorectal cancer. Many of the genes that function in the different control mechanisms governing epithelial cell turnover have been characterized, to a greater or lesser extent, has having potentially oncogenic roles. Unsurprisingly then, many of these have also been defined as potential targets for cancer therapy. The role of some of these genes and their protein products will now be discussed.

Role of Bcl-2 Family Proteins in Colorectal Cancer. Contrary to the incidence of colorectal cancer, small intestinal cancer is very rare despite its greater cellularity and higher rates of proliferation. The differential sensitivity of the small and large bowel epithelia to apoptosis has been proposed to partially contribute to this phenomenon (Potten et al., 1992). In the SI, apoptosis is an efficient mechanism for eliminating stem cells, the probable origins of neoplastic change, which have sustained genomic damage. As discussed above, apoptosis is suppressed in colonic stem cells through the expression of Bcl-2 (Merritt et al., 1995). This may be an adaptational change to cope with the harsh microenvironment of the colon, where cells are exposed to, among other things, food-derived carcinogens and high concentrations of bacterial fermentation products such as the short-chain fatty acid butyrate, which can induce IEC apoptosis (Hague et al., 1997; see next section), and in particular, the apoptosis of undifferentiated cells, as these are poor metabolizers of butyrate (Mariadason et al., 2001a).

Nonhereditary, hyperplastic conditions of the colonic mucosa are associated with an enlargement of the Bcl-2 expressing cell population within the crypts, with a majority of colonic adenomas reported as being Bcl-2 positive (Hague et al., 1994; Öfner et al., 1995; Sinicrope et al., 1995; Watson et al., 1996). Some studies suggest that Bcl-2 hyperexpression appears to be superfluous to tumor cell survival in advanced disease as the majority of adenocarcinomas of the colon do not show expression (Watson et al., 1996: Krajewska et al., 1996); this is probably due to the acquisition of other dominant mutations such as mutant p53 (Watson et al., 1996). Although Bcl-2 expression is lost, another antiapoptotic Bcl-2 protein, Bcl-w, is expressed in the majority of colonic adenocarcinomas (but not in colonic adenomas) (Wilson et al., 2000). Studies by other groups suggest that increased expression of the antiapoptotic Bcl-$X_L$ and pro-apoptotic Bak is also important in colorectal adenocarcinomas (Krajewska et al., 1996).

Cell Adhesion Signals and Cancer. Changes to cell adhesion signal transduction also play an important role in tumors of the colon. As with other tumors of epithelial origin, reduced E-cadherin expression is associated with tumor progression (Dorudi et al., 1993), increased metastatic potential (Kinsella et al., 1994), and decreased patient survival (Dorudi et al., 1995). In vitro, decreased E-cadherin expression is associated with the expression of the transcription factor Snail (Batlle et al., 2000), which binds to elements within the *E-cadherin* promoter and suppresses transcription. E-cadherin function is dependent on binding β-catenin and may regulate the levels of monomeric cytoplasmic β-catenin (as described previously). Loss of E-cadherin expression, therefore, may lead to increased free β-catenin levels.

Wnt Signaling. Accumulation of β-catenin can be readily observed in colorectal tumors. Cytoplasmic levels of β-catenin may also be enhanced, via stabilizing mutations in the region phosphorylated by GSK-3β that prevents its degradation (Morin et al., 1997); other mutations in β-catenin have also been reported (Ilyas et al., 1997). Also, mutation of the tumor suppressor *APC* results in an inability to target β-catenin for degradation and enhanced β-catenin levels (Sparks et al., 1998). *APC* +/− mice spontaneously develop intestinal adenomas during adult life, through loss of heterozygosity (Su et al., 1992).

As discussed previously, β-catenin binds to Tcf-4 to form a transcriptionally active complex that regulates the expression of a number of genes including cyclin D1 and c-myc, which drive cell proliferation and are known to be overexpressed in human tumors (for a review, see Morin, 1999).

Dysregulated expression of *Wnt*s *2*, *4*, *5a*, *6*, and *7a* has been reported in colonic tumors (Dimitriadis et al., 2001; Katoh et al., 1996; Vider et al., 1996), as has *Frizzled 10* (Terasaki et al., 2002), which codes for the Frizzled 10 receptor and is known to upregulate β-catenin signaling.

As mentioned previously, the bHLH transcriptional inhibitor Hes-1 is important in the small intestinal epithelium in regulating differentiation of endocrine cells in the intestinal epithelium. Id proteins are also bHLH transcriptional inhibitors. In addition to their inhibition of DNA binding of ubiquitous bHLH transcription factors such as E47 and tissue-specific bHLH proteins like BETA2, they also bind to a number of other cellular proteins such as pRB and the ETS proteins, SAP-1 and ELK-1 (Norton, 2000). They are known to drive proliferation and apoptosis and inhibit differentiation in a variety of cell systems. Studies show that the expression of Id is elevated in colorectal cell lines and primary colorectal tumors and that this is associated with increased proliferation (Wilson et al., 2001). These studies also show that Id dysregulation is associated with loss of wild-type p53 expression both in primary tumors and the intestinal epithelium of *p53*−/− mice. Id2 expression is also upregulated by the β-catenin/Tcf-4 pathway and can promote the anchorage-independent growth of colon cancer cells in vitro (Rockman et al., 2001).

## Effects of Commensal Bacteria on Intestinal Epithelia, in Health and Disease

The contents of the gut lumen play an important part in regulating epithelial turnover in the intestine. The major influences are diet and the microflora of the gut, both of which are responsible for the production of compounds that can regulate epithelial cell proliferation and apoptosis.

Short-chain Fatty Acids. Butyrate, propionate, and acetate are products of colonic bacterial fermentation of soluble fiber in the diet (Cummings and Englyst, 1987). Short-chain fatty acids (SCFAs) are taken up by the epithelial cells and are metabolized by β-oxidation, primarily in differentiated enterocytes, and may represent a valuable energy source for these cells (Roediger, 1982). Butyrate is also an inhibitor of histone deacetylase (HDA; Riggs et al., 1977; Sealy and Chalkley, 1978) and consequently can alter the expression of a number of genes by regulating the accessibility of transcriptional regulators to promoter sites. A recently published microarray study highlights the wide range of transcriptional regulation that takes place in response to butyrate and the synthetic HDA inhibitor, trichostatin A (Mariadason et al., 2000).

Butyrate at physiological concentrations has been shown to regulate epithelial cell turnover by influencing cell proliferation, differentiation, and apoptosis. A number of studies have shown that butyrate induces cell cycle arrest at both G1 and G2/M phases of the cell cycle and that this is related to the increased expression of the cyclin-dependent kinase inhibitors, $p21^{WAF\text{-}1/cip1}$ and $p27^{Kip1}$ (Litvak et al., 1998; Siavoshian et al., 2000; Mariadason et al., 2000). Butyrate is also able to influence the profile of IGF-binding protein secretion by colonic epithelial cells; these are important growth-regulatory molecules and so this effect may contribute to altered epithelial proliferation (Nishimura et al., 1998).

Another important effector of the cellular response to butyrate is the transcription factor Cdx2 (see previous discussion), which plays a key role in enterocyte differentiation and is upregulated by butyrate (Domon-Dell et al., 2002). Also, butyrate regulates cell signaling through the β-catenin/Tcf-1 pathway (see below; Bordonaro et al., 2002). Butyrate-induced colonocyte differentiation is associated with its ability to upregulate the vitamin D receptor (VDR) (Gaschott et al., 2001), with butyrate-induced differentiation being inhibited by a specific VDR antagonist. Many studies also show that butyrate can induce apoptosis in colorectal cell lines. This pro-apoptotic action of butyrate has been linked to the downregulation of antiapoptotic proteins such as Bcl-2 (Hague et al., 1997) and upregulation of pro-apoptotic proteins such as Bak (Hague et al., 1997; Ruemmele et al., 1999), Bax (Mandal et al., 1998), and Fas/FasL (Fan et al., 1999).

Many of the studies on butyrate action have been performed in vitro, using colorectal carcinoma cell lines. They show many cell-line-dependent

effects. For example, butyrate can enhance Tcf binding to DNA in SW620 cells; however, in HCT-116 cells butyrate downregulates Tcf expression (Bordonaro et al., 2002). Studies on the role of Bcl-2 proteins in butyrate-induced apoptosis show increased Bak but not Bax in some cell lines (Hague et al., 1997; Reummele et al., 1999), an upregulation of Bax in others (Mandal et al., 1998), and in some, no change in either Bak or Bax but downregulation of Bcl-2.

In vivo studies on butyrate action contrast sharply with in vitro results. In vivo, butyrate promotes cell proliferation and inhibits differentiation and apoptosis. Studies on guinea pig, isolated, intestinal colonic mucosa show that butyrate can inhibit enterocyte apoptosis and increased Bax expression, which occur spontaneously following isolation of the tissue (Luciano et al., 1996; Hass et al., 1997). Butyrate can also increase mucosal mass in rats (Friedel and Levine, 1992): It not known whether this effect is due to increased proliferation or decreased apoptosis. Butyrate-induced proliferation is observed in biopsies of human caecal mucosa incubated ex vivo with butyrate (Scheppach et al., 1992), rat ileum (Sakata, 1987; Kripke et al., 1989), and rat jejunum and colon (Kripke et al., 1989).

Some of the differences observed between different cell lines and between in vitro and in vivo experimental models may relate to the differentiation status of the cells being studied. CaCo-2 cells are a colorectal adenocarcinoma cell line that undergoes differentiation in vitro, over a period of 3 weeks, when maintained as confluent cultures. Undifferentiated CaCo-2 cells are highly responsive to butyrate, which can induce IL-8 secretion, increase transepithelial resistance and apoptosis (at the appropriate concentration) among other effects (Mariadason et al., 2001a). Fully differentiated CaCo-2 cells (after 3 weeks of culture at confluence) are not responsive to butyrate, an effect associated with the cells' increased ability to metabolize butyrate. HT-29cl.19A cells, which also undergo differentiation during long-term culture, give similar results but nondifferentiating SW640 cells do not (Mariadason et al., 2001a). Increased metabolism of butyrate will reduce the intracellular levels of butyrate and subsequently the transcriptional effects of butyrate related to its inhibition of deacetylation. This is shown in Fig. 3.

*Butyrate and Analogues as Sensitizing Agents for Chemotherapy.* Given its pro-apoptotic role in many colorectal cell lines, butyrate has become of great interest to researches looking for new therapeutic approaches for the treatment of colorectal cancer. Many studies now show that butyrate can specifically sensitize colorectal cancer cells to apoptosis induced by a wide range of agents including the death receptor ligands TNF-α (Giardina et al., 1999), TRAIL (Hernandez et al., 2001), and ligation of the Fas receptor (Bonnotte et al., 1998) and to agents such as 5-fluorouracil that are currently used to treat colorectal cancer (Bras-Goncalves et al., 2001).

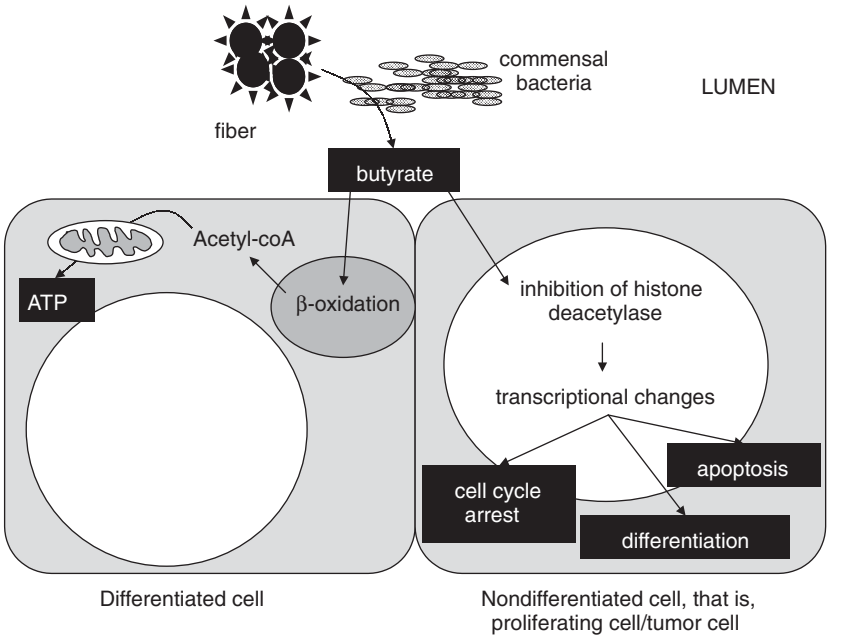


FIGURE 3. Butyrate utilization by colonocytes.

Specific analogues of butyrate have also been developed that are more pro-apoptotic than butyrate itself (Bras-Goncalves et al., 2001; Milovic et al., 2000). Cancer cells are likely to be more sensitive that mature colonocytes due to their being less differentiated (Mariadason et al., 2001a). Given the findings of the many studies on the action of butyrate, it has been proposed (not unreasonably) that intestinal levels of butyrate may act as a protective mechanism to suppress the growth of tumors in vivo. Consequently, much research has been directed at examining the prophylactic benefit of modulating intracolonic butyrate level via enteral nutrition.

Effect of Dietary Fiber and Probiotics on the Intestinal Epithelium. Dietary fiber and probiotics (live bacteria cultures that may be taken orally and have some "health benefit" to the consumer) can directly influence bacterial fermentation in the gut and hence the production of agents such as butyrate (Wang et al., 1994; Fredstrom et al., 1994) and by other, less defined mechanisms. Dietary fiber has long been held up as a healthy necessity and an important factor in suppressing colon cancer; however, many studies suggest that fiber may be more of a risk than a benefit. For example, increasing the fiber content of diet can increase colon mass, via increased crypt cell production; this is amplified by increased crypt branching and fission, which serves to increase total stem cell numbers in

the colon (McCullogh et al., 1998). The controversy surrounding the role of dietary fiber is reviewed in a commentary by Goodlad (2001).

Lactobacilli also have positive effects on epithelial cell proliferation in both rats and mice (Ichikawa et al., 1999; Thoreux et al., 1998), although as yet there is no direct evidence to link their effects with altered levels of SCFAs. The proliferative effects of probiotics on the intestinal epithelium have led to their use as therapeutic agents to aid the restitution of the intestinal mucosa following injury, for example, in chronic inflammatory conditions such as Crohn's and ulcerative colitis (Madsen, 2001; Gassull and Cabre, 2001).

## Methods/Technology

The methodologies used to study epithelial cell turnover in the intestine are much the same as for any other tissue. The highly organized structure of the tissue does, however, have the specific advantage of allowing the analysis of cellular events on a positional basis within the cellular hierarchy.

The techniques most commonly applied to studying apoptosis in the intestinal epithelium are simple assessment of cellular morphology using light; fluorescence or electron microscopy, with which one can readily distinguish apoptotic cells on the basis of cell shrinkage; loss of cytoplasm; plasma membrane blebbing; and nuclear condensation and fragmentation. These forms of analysis may be complemented by labeling cells for evidence of biochemical change such as labeling of DNA strand breaks, or used in parallel with the assessment of proliferation markers such as labeling with tritiated thymidine or bromodeoxyuridine, expression of nuclear proteins such as Ki67, and quantitation of mitotic figures. These methods have been extensively reviewed elsewhere and readers are referred to Merritt et al. (1996) and Wilson and Potten (1999) for a full description of the various methodologies. These classical techniques and imaging systems have both been refined over the years, but one of the major improvements has been in the use of more sophisticated animal models such as the ROSA28/sv190 chimeric mouse and transgenic and knockout mice, many of which have been discussed in this chapter (Wong et al., 2002). The culture of primary human intestinal biopsies is slowly being developed as a technique, but is still not universally established in all gastroenterology laboratories.

Like many other areas of cell biology, new techniques such as laser capture microdissection and microarray analysis of gene expression are being applied to the study of intestinal epithelium. The response of colorectal cancer cells to butyrate (Mariadason et al., 2000) and the study of differential gene expression along the anterior–posterior axis of the gastrointestinal tract (Bates et al., 2002) are just two recent examples of the application of microarray analysis in gastrointestinal biology.

## Conclusion

The current vogue, reiterated by many articles of this type, is the expectation that our understanding of the molecular and genetic controls which regulate intestinal stem cells and IEC apoptosis, proliferation, and differentiation will grow rapidly over the next few years through the utilization of knowledge from genome sequencing projects and the application of high throughput genomic and proteomic technologies.

This may be true, but so far the majority of our knowledge has resulted from in vivo studies in mice, examining responses to epithelial injury, and also through the use of transgene and knockout models. Maybe over the next few years we will come to know precisely what determines stem cell status and hierarchical status in the intestinal epithelium. Also, perhaps we will learn how to manipulate IEC apoptosis and proliferation more directly at a molecular level, in order to contribute to improved treatment of intestinal disease.

Web Resources. For everything you always wanted to know about wnts, see ⟨http://www.stanford.edu/~rnusse/wntwindow.html⟩. To review *Science* magazine's online STKE (Signal Transduction Research Environment), go to ⟨http://stke.sciencemag.org/index.dtl⟩ (requires subscription payment for full access).

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CHAPTER

10

# CELL DEATH, AGING PHENOTYPES, AND MODELS OF PREMATURE AGING

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The idea that cell death may be an important factor in aging is not new, as several comprehensive reviews on this subject have been published during the past five years (Warner et al., 1997; Zakeri and Lockshin, 2001; Joaquin and Gollapudi, 2001; Zhang and Herman, 2002a). Although it seems obvious that the loss of cells from any particular tissue may ultimately compromise the function of that particular tissue and/or its ability to fulfill the needs of the whole organism, the relative vulnerability of different tissues to net cell loss is not so clear. This uncertainty stems from our lack of precise knowledge about how much intrinsic cell death occurs in the absence of overt pathology, the potential for replacement of lost cells, the amount of excess functional capacity in each tissue, and how any of these parameters might change with age. Although it is well known that cell loss in postmitotic tissues has particularly tragic consequences because of the very low rate of cell replacement in these tissues, there is much that remains to be learned about the death of cells and their replacement during aging in other tissues as well.

Cell death in vivo occurs by a variety of mechanisms depending on the nature and magnitude of the insult experienced by the cell. In their recent review, Zakeri and Lockshin (2001) briefly described apoptosis (also referred

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to as programmed cell death), necrosis due to acute injury, and autophagic cell death in which damaged cells are engulfed by phagocytes. They also pointed out that there are not always clear borders among these three processes. Recently, Sperandio et al. (2000) proposed the existence of an apoptosis-like form of programmed cell death in some cases of neurodegeneration that they called paraptosis. It differs from apoptosis in its lack of response to caspase inhibitors, and the absence of nuclear fragmentation and caspase-3 processing. It mainly differs from necrosis in its requirement for RNA and protein synthesis, and it is induced by expression of the receptor for IGF-I or caspase-9.

The purpose of this review is not to exhaustively rereview this entire area of research. Zakeri and Lockshin (2001) summarized the molecular features of receptors, inducers and cell death pathways in their review, and briefly discussed the roles of cell death in immune function, neurodegenerative disease, and cancer. They also discussed the effect of replicative senescence of cultured cells on their vulnerability to apoptosis, which remains a controversial area of research. Several investigators have reported that senescent cells are resistant to apoptosis in vitro (Wang, 1995; Spaulding et al., 1999), whereas Pignolo et al. (1994) reported that there is also substantial loss of cells from senescent cultures. The effect of replicative senescence on susceptibility to apoptosis in vivo thus remains unclear, although it is clear that postmitotic cells such as neurons and cardiomyocytes do die in vivo, and do at least sometimes by apoptosis (see below). This brief review will focus primarily on a few recent publications that implicate cell death as a risk factor for various aging phenotypes and pathologies in mice, and is intended to be more speculative than comprehensive.

## Does Apoptotic Potential Change with Aging?

We postulated in 1995 that the ability to undergo apoptosis in rodent liver may decrease with age (Warner et al., 1995), and suggested that this might be a factor in increasing tumorigenesis with increasing age. This hypothesis was based primarily on the positive association between aging and tumorigenesis, and the ability of caloric restriction to attenuate both, while upregulating apoptosis in rat and mouse liver (James and Muskhelishvili, 1994; Grasl-Kraupp et al., 1994). Caloric restriction also increases the expression of both Fas and Fas-ligand in mouse lymphocytes, which may contribute to the increased life expectancy of calorically restricted mice (Avula et al., 1999). However, these are weak, indirect arguments, and little data on age-related changes in the rate of apoptosis in specific tissues are available.

One of the main problems is that it is not clear what to measure, and what the measurements would mean. Can one evaluate apoptotic potential by measuring any one component or several components of an apoptotic pathway? This latter approach was used by Lacelle et al. (2002), who

measured caspase mRNA levels in peripheral blood mononuclear (PBM) cells taken from members of a Taiwanese population of individuals between 10 and 102 years old. The levels of caspase-1 and caspase-3 mRNAs measured in this survey did increase with age, while the levels of caspase-8 mRNAs peaked at about 80 years of age, but then fell dramatically. Whether these mRNA levels reflect the amount of apoptosis actually going on in the PBM cells, or whether these differences are obtained in any other tissue, is not known. The mRNA levels of Fas, an apoptosis inducer, have also been shown to increase with age in rat liver (Higami et al., 1997).

A second approach is to look at the intrinsic level of cells undergoing apoptosis in tissues as a function of age, but such data might also be hard to interpret. It would not be known for sure whether the differences observed in the number of apoptotic bodies counted reflect different rates of apoptosis, that is, the length of time required for a cell to die, or a different sensitivity to the damaging events that induce apoptosis. Using such an approach, Barnes et al. (1999) reported that apoptotic cells decrease with age in rat seminiferous tubules, and Holt et al. (1998) reported a slight decrease in the apoptotic index in small intestine epithelial cells between 4 and 24 months of age, but a 5-fold increase in this index in old rats, but not young rats, during caloric restriction. A similar increase was observed in the colon.

Suh et al. (2002) also employed such an approach, but challenged young and old rats with the carcinogen methyl methanesulfonate (MMS), and then looked at the number of cells undergoing DNA fragmentation in liver tissue, both before and 2 hours after MMS injection. This genotoxic stress induced substantial apoptosis in the livers of 2-month-old rats, but almost no apoptosis in the livers of 26-month-old rats, even though DNA damage in the liver is known to be higher in old mice than in young mice (Gaubatz and Tan, 1997). Thus, even though DNA damage is presumably also occurring in the old rats, the liver cells appear to be resistant to MMS-induced apoptosis. The authors suggest that the signaling systems involved in liver cell apoptosis are downregulated with increasing age, thereby contributing to age-related carcinogenesis in the rat.

Thus, this question of whether apoptotic potential increases with age is unresolved, at least partly because of the lack of suitable strategies to measure apoptotic potential. Furthermore, it would be difficult to justify extrapolating results obtained with any one tissue such as liver, to any other tissue because of the multiplicity of factors that might be involved in maintaining tissue homeostasis in different tissues.

## Cell Death in Postmitotic Tissues

In an earlier review (Warner et al., 1997), we briefly summarized the evidence for loss of neurons by apoptosis in a variety of neurodegenerative diseases.

Nevertheless, this conclusion remains controversial, and the on-going debate has been recently summarized by Jean Marx (2001) in *Science*. Cotman and Anderson (1995) reported that TUNEL staining in Alzheimer's patients' brains is markedly greater than that found in the brains of people who died from other causes, but the results suggested that too many neurons were dying, considering the slow onset of this disease. Thus, more recent approaches have focused on determining the level of active caspases, the proteases that actually kill the cell. The main executioner is thought to be caspase-3, with caspases-8, -9, and -12 playing important supporting roles in the apoptotic cascade. Using antibodies specific for the active form of caspase-3, Su et al. (2001) showed that caspase-3 is elevated in Alzheimer's brain neurons at a level of about 0.1% of the total neurons surveyed. Caspase-8 is also activated in the Alzheimer's brain, and may be involved in activating caspase-3 (Rohn et al., 2001), whereas Nakagawa et al. (2000) have reported that caspase-12 is required for Aβ-induced apoptosis of cultured cortical neurons. These results indicate that apoptotic pathways are elevated in the brains of Alzheimer's patients, and may be a major factor in Aβ neurotoxicity.

Piero Anversa (1998) has reviewed the role of myocyte death in heart failure, and has estimated that about $5 \times 10^7$ myocytes are lost from the heart per year, but that myocyte regeneration compensates for most of this loss, and may even result in hypertrophy. Anversa raises the question of whether apoptosis is an important factor in heart failure, or whether necrosis is the major factor. Guerra et al. (1999) measured the frequency of apoptosis and necrosis in the failed hearts of patients undergoing heart transplantation and reported that necrosis exceeds apoptosis by 7-fold in both men and women, but that both are about 2-fold higher in men than in women. Apoptosis is 35- and 85-fold higher in diseased hearts compared to control hearts in women and men, respectively. Increases in necrosis represented only one-third of these values. These results suggest that both necrosis and apoptosis contribute to heart failure, but that apoptosis is selectively more elevated than necrosis.

A critical issue, however, is whether these cells can be replaced by the proliferation of appropriate stem cells. Orlic et al. (2001) have shown that exogenous bone marrow cells can repopulate the infracted heart and generate myocardium. Although many questions remain (Sussman, 2001), this result suggests that stem cell therapy for heart failure remains a possibility.

## Cell Death in Mutant Mouse Models of Aging

### $p66^{shc}$ Function

The concept that cell death in mitotic tissues is an important factor in mammalian aging and longevity is strengthened by the observation of

Migliaccio et al. (1999) that a mutation in the *p66$^{shc}$* gene increases the ability of mouse cells to resist apoptosis induced by hydrogen peroxide or UV light, and increases life expectancy of the mice by about 30% in the absence of an oxidative challenge. The p66$^{shc}$ gene product is part of a signal transduction pathway that is activated by reactive oxygen species and leads to apoptosis. Although we do not yet know enough to place this finding in an aging perspective, Zhang and Herman (2002b) point out the following: "Our overall health relies to a great extent on the proper balance between normal removal of damaged cells via apoptosis and proliferation of the cells that comprise our body. Tipping the delicate balance towards either side may cause diseases and hamper successful aging". (p. 563). However, in this p66$^{shc}$ mutant mouse, longevity is enhanced. Thus, it is clear we do not yet have a coherent picture of the connections between cell death and replacement, and aging in mammals. To study this, premature aging models may be as informative as models of extended longevity, as discussed below.

## p53 Function

It is well documented that the p53 tumor suppressor protein plays a critical role in several cellular phenomena that have implications for aging. The p53 protein regulates the transcription of genes involved in both cell cycle regulation and survival (Levine, 1997), and is required for radiation-induced and oxidative-stress-induced apoptosis (Lowe et al., 1993). Cellular stresses such as radiation or oxidative stress activate expression of the *p53* gene that may then induce expression of the *p21* gene whose gene product leads to growth arrest until repair takes place, or to apoptosis if the cellular damage is too great to be repaired (Duttaroy et al., 1997). Replicative senescence results from the p53 induction of p21 in response to various stresses, including telomere shortening.

Thus, the p53 protein plays a critical balancing role in the cell by controlling both cell proliferation and apoptosis. p53 deficiency leads to early induction of cancer in both mice (Donehower et al., 1992) and humans (Hollstein et al., 1994), and human tumor cells frequently contain mutant p53 genes. However, Tyner et al. (2002) recently reported the unexpected result that deletion of a particular part of the *p53* gene in mice can lead to a gain in its tumor suppression function, and mice containing one good *p53* allele and one such mutant allele (*p53$^{+/m}$*) are actually resistant to cancer. However, instead of living longer because of their resistance to cancer, these mice appear to age prematurely, and die about 20 to 25% earlier than normal mice even though they have far fewer tumors. The p53$^{+/m}$ mice exhibited generalized organ atrophy, reduced stress resistance and osteoporosis, whereas subcutaneous fat, dermal thickness, and wound healing were all reduced in these mutant mice.

These authors hypothesized that these early aging phenotypes resulted from a failure to maintain adequate cell numbers, particularly in those tissues dependent on stem cells for replacement of cells lost through normal cell turnover. They were unable to conclude whether this is due to excessive apoptosis, or inadequate cell replacement in these tissues, but they suggested that "an aging-related reduction in stem cell proliferation may have a more important role in longevity than previously recognized recognized" (Tyner et al., 2002, p. 50). In these mice, cell loss could be particularly acute because of hyperactive pro-apoptotic p53 function. Thus, p53 function in mammals now becomes another possible example of antagonistic pleiotropy in aging (Campisi, 2002); although p53 function represses cancer early in life, it may compromise replacement of cells by stem cell proliferation late in life (Warner, 2002), thus leading to slowly atrophying tissues with increasing age.

An important linkage between aging and p53 function is also supported by recent findings on the role of the sir2 protein in aging and regulation of p53 activity. The active form of p53 is both phosphorylated and acetylated. The mouse sir2 gene product functions as an NAD-dependent p53 deacetylase activity that preferentially removes the acetyl group on lysine 382 of p53 in vitro (Vaziri et al., 2001). Other studies have shown that overexpression of the *sir2* gene in yeast and nematodes extends their life span (Kaeberlin et al., 1999; Tissenbaum and Guarente, 2001). Such overexpression presumably leads to attenuation of both p53 function and apoptosis, and increased proliferative potential (Luo et al., 2001). As is often the case, this is an oversimplified model, as Kang et al. (2002) have shown that pharmacologic inhibition of histone deacetylase activity in fruit flies by phenylbutyrate also extends life expectancy. Thus, there may be an optimal level of histone deacetylase activity, subject to a number of regulatory influences, that differs among organisms.

## DNA Damage/XPD Function

De Boer et al. (2002) recently created transgenic mice carrying a mutation in the *XPD* gene that codes for a DNA helicase involved in both transcription and repair of DNA damage. The mutation does not knock out the helicase activity, but significantly reduces it. As a result, these mice have substantially impaired transcription and mildly impaired DNA repair. The phenotype of these transgenic mice includes not only osteoporosis, loss of female fertility, and premature graying of hair, but also reduced life span (median life expectancy of <12 months vs. 24 months for the controls). These mice appear to be normal at birth and remain normal for about 4 months, although they are 10 to 20% smaller. However, by 6 months of age the above aging phenotypes begin to appear. The molecular basis for the age-related onset of these aging-like phenotypes is not known, but de Boer et al. suggest that the

failure to repair DNA damage and allow transcription to proceed triggers programmed cell death, "leading to functional decline and depletion of cell renewal capacity" (p. 1279).

### What Can These Mouse Models Tell Us about Aging?

Although we do not know what a "pure" aging phenotype unencumbered by age-related disease would look like, most gerontologists agree that the aging phenotype includes failure to maintain and/or return to homeostasis after exposure to a stress, no matter what system they happen to be studying. Until recently, there has been little direct experimental evidence to back up this assumption. Homeostatic failure in this context implies the inability to maintain an adequate number of normally functioning cells in each tissue with increasing age. The respective authors of these two papers (Tyner et al., 2002; de Boer et al., 2002) speculate that stem cell populations, and their ability to proliferate in order to replace cells lost through normal wear and tear, may play a more important role in maintaining tissue homeostasis during aging than was previously appreciated, and may therefore play a critical role in the regulation of life expectancy in mammals. Hasty and Vijg (2002) also make such a suggestion in their review of the results reported by de Boer et al. (4). The dynamics of cell turnover during aging has not been comprehensively studied on a tissue-by-tissue basis, possibly because cell turnover rates are normally so low that these dynamics would be hard to measure with any accuracy. Whether the p53 and XPD mutants will provide an opportunity to measure accelerated turnover rates, and relate these to tissue homeostasis in these mutants remains to be seen.

## Human Premature Aging Syndromes

Some comparisons of the p53 and XPD mouse mutant phenotypes with those of several segmental progeroid syndromes in humans are summarized in Table 1. Two of the most well known human progeroid syndromes are Hutchinson–Gilford syndrome (HGS), also referred to as progeria, and Werner syndrome (WS). HGS patients appear normal at birth, but by the end of their second year growth begins to slow and loss of hair and subcutaneous fat begin (De Busk, 1972). Although their intellectual development is normal, these children reach a height of only about 3 feet and a weight of about 35 pounds, and usually die of cardiovascular complications at an average age of 13 years. The syndrome is very rare (about 1 per 10 million births), and the genetic defect in HGS is not known, although it is usually assumed that HGS is due to an autosomal dominant mutation, possibly arising during germ cell production or development. The short stature and

TABLE 1. COMPARASON OF SEVERAL MOUSE MUTANTS WITH HUMAN PROGEROID SYNDROMES

| | Mouse Mutant | | Human Syndromes | | |
|---|---|---|---|---|---|
| Phenotype | p53 | XPD | HGS | BS | WS |
| Biochemical defect | Hyperactive p53 | Lack a DNA helicase | Unknown | Lack a DNA helicase | Lack a DNA helicase |
| Life expectancy | 100 weeks | <50 weeks | 13 years | 25 years | 50 years |
| Percentage of normal | 80% | <50% | 17% | 33% | 67% |
| Growth failure begins | Late in life[a] | Month 4–6 | Year 2 | Before birth | Year 15–18 |
| Cancer risk | Reduced | ? | Normal ? | Elevated | Elevated |
| Osteoporosis | Premature | Premature | No ? | No ? | Premature |
| Subcutaneous fat loss | Yes | ? | Yes | ? | Yes |
| Slow wound healing | Yes | ? | ? | ? | Yes |

[a] General organ failure occurs by 18–24 months.

musculoskeletal abnormalities are more consistent with developmental abnormalities than premature aging, although fibroblasts taken from HGS patients have short telomeres and little replicative potential remaining, suggesting the replicative life span of these cells may have been compromised by excessive apoptosis and cell replacement early in life. The short stature and developmental abnormalities might then be due to insufficient cell replacement from the various stem cell reservoirs with increasing age. However, other than short telomeres, there is no strong evidence for genetic instability in HGS that might trigger such an early and continuing apoptotic response.

WS patients also appear normal at birth, and a diagnosis of WS is usually not made until puberty, when growth begins to slow down (Martin and Oshima, 2000). This is followed by premature graying of hair, atrophy of various tissues (particularly reproductive tissues and skin), type 2 diabetes, atherosclerosis, and osteoporosis. Most striking, however, is the genetic instability that accompanies WS, so WS patients are at high risk for neoplasia. This is consistent with the discovery that the *WRN* gene codes for a protein with DNA helicase (known as a recQ helicase) and $3' \rightarrow 5'$ exonuclease activities (Gray et al., 1997; Huang et al., 1998). Thus, this protein may be involved in any one or all of the following: replication, repair, recombination, and transcription. Fibroblasts isolated from WS patients have short life spans, but longer than that of HGS fibroblasts. WS patients usually die of either cancer or myocardial infarction at a median age of about 47 to 48 years.

In contrast to HGS and WS, patients with Bloom syndrome (BS) do not appear normal at birth, but are born small, and remain smaller than normal throughout their short life, with death usually occurring in their twenties due to cancer. The defective gene (*BLM*) associated with Bloom syndrome also codes for an enzyme with a recQ-like DNA helicase activity, but not exonuclease activity. Several speakers at the recent Keystone symposium on "DNA Helicases, Cancer and Aging" (March 12–17, 2002) suggested that the small stature of Bloom syndrome patients may result from excessive cell death, even in the fetal stage of life. Because BS is characterized by genomic instability, particularly sister chromatid exchanges, such cell death may be triggered by the accumulation of replication intermediates (Bischof et al., 2001) that either induce cell death or lead to illegitimate recombination. The growth defects associated with these three human syndromes appear not to be caused by growth hormone deficiency (Laron, 2002).

The preliminary results with Bloom syndrome raise the issue of whether excessive cell death also contributes to the phenotype of Werner, Hutchinson–Gilford, or Cockayne syndrome, all of which are characterized by short stature, and/or to other segmental progeroid syndromes. On the other hand, the increased susceptibility to cancer might indicate that decreased apoptosis is occurring in WS patients (Campisi, 2002). These questions seem to deserve increased attention in future research on the roles of

cell death in aging and development of various aging phenotypes, and this may also tell us something about p53 function and aging, as Yang et al. (2002) have reported that p53 may also play a role in regulating the activity of the DNA helicases associated with Werner and Bloom syndromes.

## Discussion

Six mouse mutants with increased life expectancy have so far been identified; four of these are dwarf mice and the reduced growth rate observed in these mice is due either to growth hormone deficiency or the inability to respond to growth hormone (Bartke et al., 2001). Thus, growth failure per se is clearly not an indication of premature aging. Another class of mouse mutants is apparently normal at birth and the pups initially grow normally, but their growth rate eventually slows and the mice appear to age prematurely. The collective phenotype of these mice (growth failure, osteoporosis, loss of subcutaneous fat, small organ size) has been interpreted as indications of excessive cell death and/or inadequate cell replacement with increasing age, although this has not been demonstrated directly. If this hypothesis is true, it suggests that cell turnover may be a more important factor in aging and the development of aging phenotypes than was previously appreciated, and suggests the need for better methods to measure intrinsic rates of various forms of cell death as a function of tissue and age.

Some parallels between these mouse models and some human segmental progeroid syndromes suggest the possibility that failure to maintain tissue homeostasis may have a role in the premature aging phenotypes seen in these human syndromes. Determining whether this is so, and what causes it, would appear to be a fruitful area of research on the molecular basis of aging in humans. It would be particularly interesting to determine whether cell death and replacement are factors in "timing" the growth failure that occurs in various human segmental progeroid syndromes.

Note added in proof: The recent discovery that HGS is caused by mutations in the gene for lamin A/C is consistent with the above discussion (Eriksson et al. Recurrent de novo point mutations in lamin A cause Hutchinson-Gilford progeria syndrome, *Nature* 423: 293–298 (2003)). Lamin A is a major component of the nuclear envelope and the nuclei in HGS cells are visibly mishapen, suggesting these cells may be susceptible to apoptosis.

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SECTION

III

# How Cell Death Is Carried Out

CHAPTER

11

# SURVIVAL FACTORS

DAVID L. VAUX

Survival factors are a convenient place to begin a description of mechanisms of apoptosis for historical reasons. The gene for Bcl-2 is involved in chromosomal translocations found in the common lymphoid malignancy, follicular lymphoma, which suggested that it may be a cancer gene. Experiments designed to determine how Bcl-2 caused lymphoma revealed that it did not act like other oncogenes by stimulating cell proliferation. It turned out to be an apoptosis inhibitor, the first component of the mechanism for physiological cell death to be identified in any organism (Vaux et al., 1988). These experiments also indicated that inhibition of cell death could lead to cancer.

Although experiments such as these made it clear that Bcl-2 could inhibit physiological cell death, for a number of years it was unclear how Bcl-2 worked, or what the other components of the apoptotic mechanism might be. Meanwhile, experiments in *C. elegans* had shown that the nematode had a genetic program that was specific for developmental programmed cell death, and had no other role in worm physiology (Ellis and Horvitz, 1986). However, at this stage the genes had not been cloned and it was unclear whether programmed cell death in the worm had anything in common with apoptosis in mammals.

That cell death studies in *C. elegans* were indeed relevant to mammalian apoptosis became clear when transgenic expression of human *bcl-2* in *C. elegans* was shown to prevent most of the programmed cell death during worm development (Vaux et al., 1992). Subsequently it was shown that *bcl-*

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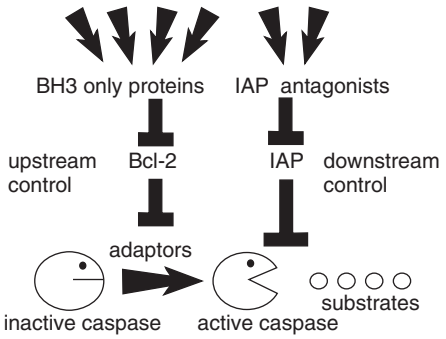


FIGURE 1. Survival factors control apoptosis either by preventing caspases from activating, or by binding to and inhibiting caspases after they have been activated. In this way, they can integrate and control many different signals that determine whether a cell kills itself or remains alive.

*2* and *ced-9* had similar sequences, and human *bcl-2* could prevent cell death in *ced-9* mutant worms (Hengartner and Horvitz, 1994). These experiments showed that programmed cell death and apoptosis are one and the same process, and possibly even more important, suggested that just as CED-9 acted by inhibiting the pro-apoptotic molecules CED-4 and CED-3 in the worm, Bcl-2 would prevent apoptosis of mammalian cells by inhibiting proteins homologous to them. We now know that CED-4 is an adaptor protein similar to mammalian Apaf-1 (Zou et al., 1997), and CED-3 is a caspase (Yuan et al., 1993).

In addition to acting prior to caspase activation, survival factors have been identified that act by inhibiting activated caspases (Fig. 1). The first survival factors that acted as protease inhibitors were derived from viruses. CrmA from cowpox virus was identified as an inhibitor of caspase 1 (Ray et al., 1992), the protease responsible for processing interleukin 1β. Later experiments showed that it could also strongly inhibit caspase-8 (Zhou et al., 1997), and thereby inhibit apoptosis triggered by ligation of receptors related to the TNF receptor, sometimes called "death receptors." Baculoviruses were found to encode two different kinds of caspase inhibitors, p35 and inhibitor of apoptosis (IAP) proteins (Clem et al., 1991; Crook et al., 1993). p35 is able to bind to and inhibit caspases from many different species, including worms, insects, and mammals. The viral IAPs allowed the subsequent identification of cellular IAP homologues in insects and vertebrates. Biochemical and structural studies have shown how these three different proteins directly bind to and inhibit active caspases.

## Upstream Inhibition of Caspase Activation: The Bcl-2 Family

### Three Types of Bcl-2 Family Proteins

Mammals have over a dozen different proteins that resemble Bcl-2, and these can be grouped into three classes (Fig. 2). A1, Mcl-1, Bcl-x, Boo, and Bcl-w are, like Bcl-2, antiapoptotic proteins that can prevent cells from killing themselves, thus allowing them to reproduce, even after pro-apoptotic insults such as growth factor deprivation, or exposure to radiation, steroids, or toxins.

Bax, Bak, and Bok, on the other hand, are pro-apoptotic, increasing the likelihood that cells exposed to an insult will kill themselves, and yet they have a structure comprised almost entirely of alpha helices that is very much like that of the antiapoptotic Bcl-2 family members. These pro-apoptotic proteins also have several of the four "Bcl-2 homology" (BH) domains that are seen in the antiapoptotic proteins. The third class of Bcl-2 family proteins, which includes Bid, Bim, Blk, Bmf, Puma, Noxa, Hrk, Bad, and Bik, is pro-apoptotic, but their similarity to Bcl-2 is restricted to possession of a single BH3 domain. Hence, they are often referred to as "BH3-only" proteins.

### BH3-Only Proteins: Bcl-2 Antagonists

Most of the regulation of the antiapoptotic proteins, including CED-9 in *C. elegans*, and Bcl-2 and Bcl-x in mammals, occurs posttranslationally, by direct binding of pro-apoptotic BH3-only proteins (Huang and Strasser, 2000). For example, genetic analysis in *C. elegans* has shown that the BH3-only protein EGL-1 inhibits the antiapoptotic activity of CED-9, and CED-9 bearing a mutation in its BH3 binding groove is not efficiently antagonized by EGL-1 (Conradt and Horvitz, 1998). In mammalian systems structural studies have shown the BH3 alpha helix of Bad binds to a groove on the surface of Bcl-x (Kelekar et al., 1997; Sattler et al., 1997) (Fig. 3). Similarly, Bcl-x and Bcl-2 are inhibited by binding of BIM, Noxa, Bad, and the like. via their BH3 domains (reviewed in Adams and Cory, 2001).

The BH3-only proteins themselves are regulated in a variety of ways that allow a cell to respond to a large variety of apoptotic stimuli. *Egl-1* in *C. elegans* is under transcriptional control. So are the mammalian BH3-only proteins Noxa and Puma, whose expression is induced by p53. Bad is regulated by phosphorylation, so that the phosphorylated form is sequestered by 14-3-3 proteins, whereas its unphosphorylated form can bind and antagonize Bcl-x. Bim is sequestered away from antiapoptotic proteins into microtubules via its association with dynein light chain, such that it can be released following apoptotic stimuli such as treatment with Taxol. Bmf associates

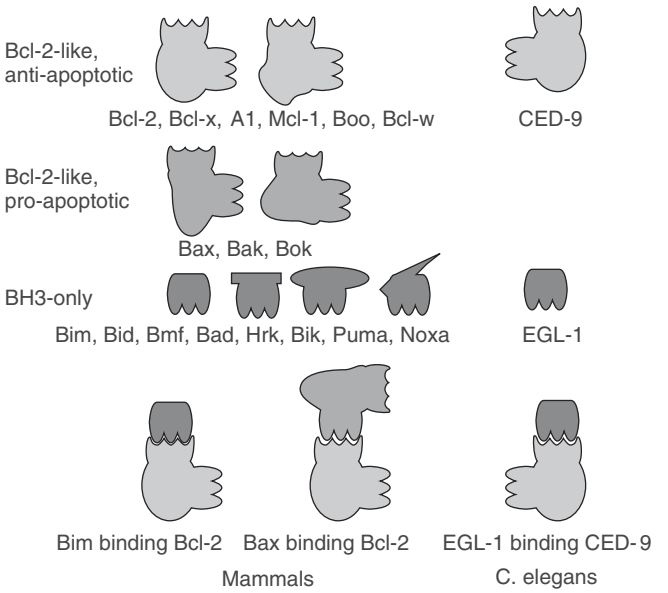


FIGURE 2. Three kinds of Bcl-2 family members. The subgroup that includes Bcl-2 itself inhibits apoptosis, whereas the Bax group promotes apoptosis. The "BH3-only" group, which resembles the others only by possessing a BH3 alpha helix, is also pro-apoptotic. Interactions between Bcl-2 family members involve binding of the BH3 helix of one protein (represented by three small bumps) to a groove on the surface of another protein. No Bax-like pro-apoptotic protein has been identified in *C. elegans*.

with the actin cytoskeleton, allowing it to promote apoptosis in response to loss of attachment ("anoikis") (Puthalakath et al., 2001). Bid is activated by proteolysis, either by caspase-8, caspase-3, or following attack by a cytotoxic T cell, by granzyme B.

## Pro-Apoptotic Bcl-2 Family Members Bak and Bax

These proteins resemble Bcl-2 much more than the BH3-only proteins do, because in addition to bearing BH3 domains, they also have BH1 and BH2 domains. However, like the BH3-only proteins, they are pro-apoptotic. Exactly how they work remains controversial. What is clear is that Bax and Bak are essential for apoptosis in some, but not all, circumstances, because mice lacking both Bax and Bak retain the webbing between their digits, accumulate lymphoid cells, and have increased populations of certain kinds of neurons (Lindsten et al., 2000). *bax/bak* double mutant cells are resistant to

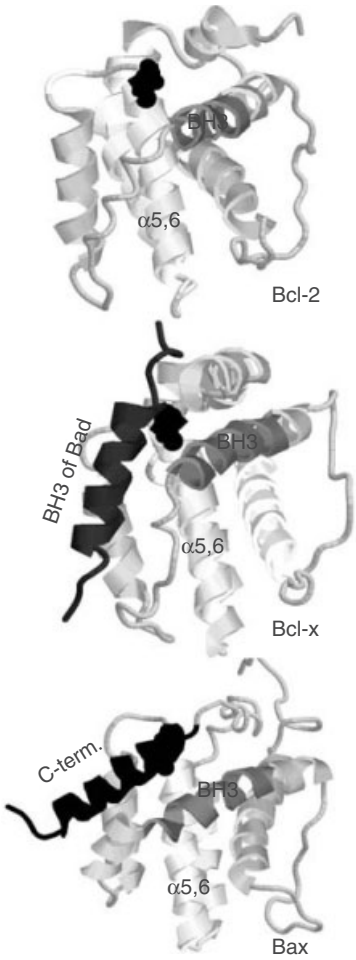


FIGURE 3. Structures of Bcl-2 family members. Top: structure of Bcl-2. Bcl-2 is almost entirely comprised of alpha helices. It has been proposed that the two central ones (α5 and α6) insert into membranes to form a pore. The BH3 region is colored gray, and a conserved glycine residue that lies at the end of the groove into which the BH3 of other family members can bind is colored black. Middle: structure of Bcl-x with bound BH3 peptide of Bad (dark gray). Bottom: structure of Bax. Note the overall similarity to Bcl-2. The groove in Bax is occupied by Bax's hydrophobic C-terminal tail (black).

many apoptosis inducers, but not that triggered by death receptors (Wei et al., 2001).

In general terms there are two models for how Bax and Bak function (reviewed in Adams and Cory, 2001). One is that they act like the BH3-only

proteins by directly binding to and antagonizing antiapoptotic Bcl-2 family members such as Bcl-2 and Bcl-x, via their BH3 domains. The other possibility is based on the fact that Bax and Bak, like Bcl-2 and Bcl-x, are comprised almost entirely of alpha helices, which is reminiscent of the bacterial channel or pore-forming proteins diphtheria toxin and colicin (Muchmore et al., 1996). This has led to speculation that these proteins oligomerize, inserting their central two alpha helices into cell membranes to form channels or pores (Jurgensmeier et al., 1998). According to this model, these channels directly or indirectly allow release of cytochrome c, which binds to the adaptor protein, Apaf-1, causing it to activate caspase-9. Bax or Bak oligomerization might be triggered by a BH3-only protein such as Bid (Eskes et al., 2000; Korsmeyer et al., 2000). Although some propose that Bax and Bak are capable of forming pores on their own, others think that they form channels in association with mitochondrial proteins such as the adenine nucleotide translocator (ANT) or VDAC (Zamzami et al., 2000).

The crystal structure of Bax revealed that an alpha helix in its C-terminal tail is folded over to occupy the groove on the surface of Bax into which the BH3 helix of other proteins might bind (Suzuki et al., 2000) (Fig. 3). The significance of this finding is not certain, but it may allow regulation of the localization of Bax, because the C-terminus would only be free to interact with membranes once it was displaced from this groove, and binding of other BH3 proteins or oligomerization of Bax would be prevented until the C-terminal tail was displaced. When cells are exposed to an apoptotic stimulus, Bax is often observed to translocate from the cytosol to mitochondrial membranes (Nechushtan et al., 1999). This may occur when the apoptotic stimulus frees a BH3-only protein, which binds to Bax, displacing its C-terminal tail, allowing it to associate with membranes.

The structures determined for Bcl-2 and Bcl-x were of proteins lacking the C-terminal helix, so it is possible that it lies in the BH3-binding groove of these proteins as it does in Bax.

## Bcl-2 Function

Experiments in which the worm Bcl-2 homologue CED-9 and the worm Apaf-1-like adaptor protein CED-4 were expressed in yeast or insect cells have shown that these proteins can bind directly to each other, and this has led to the simple model that CED-9 inhibits programmed cell death by binding to CED-4, thereby stopping it from binding to the caspase CED-3 and activating it (Hengartner, 2000). Because human Bcl-2 can function in *C. elegans*, and even function in CED-9 mutants, it seems likely that Bcl-2 and CED-9 act in a similar way (Vaux et al., 1992; Hengartner and Horvitz, 1994). Therefore, in mammalian cells Bcl-2 would presumably also bind directly to an adaptor such as Apaf-1.

Unfortunately, the situation seems to be either different or much more complicated in mammalian cells than in the worm. Initial reports that the CED-4 homologue Apaf-1 could bind directly to the mammalian Bcl-2-like antiapoptotic protein Bcl-xl could not be confirmed, and Bcl-2 can still inhibit apoptosis in cells in which Apaf-1 has been knocked out (Haraguchi et al., 2000). Bcl-2 must therefore be able to function independently of Apaf-1, but exactly how it does so is one of the most contentious areas of cell death research.

There are now, in essence, two models for how antiapoptotic Bcl-2 family members work. One is that they act as antagonists of yet-to-be-discovered CED-4-like adaptor proteins, that is, just as CED-9 appears to work in *C. elegans*. According to this model, Bcl-2 keeps a CED-4-like adaptor protein from activating caspases (Hengartner, 2000). If there is insufficient Bcl-2, or it is antagonized by Bax, Bak, or BH3-only proteins, the adaptors are free to activate caspases, which as well as cleaving proteins in the cytosol digest proteins in the mitochondrial membranes, leading to release of proteins such as cytochrome c, resulting in cell death. In this model, secondary mitochondrial events such as the release of cytochrome c are caspase-dependent and not essential for the cell to die, but may amplify and hasten full caspase activation.

The other model is that within mitochondrial membranes, Bcl-2 and Bcl-x directly, or indirectly via pro-apoptotic proteins such as Bax and Bak, prevent release of cytochrome c from the mitochondria. In this model, Bcl-2 and Bcl-x do not interact with a CED-4-like protein such as Apaf-1; rather, Apaf-1 is bound by cytochrome c after it is released from the mitochondria, and Apaf-1 then activates caspase-9 (Wang, 2001; Yang et al., 1997). According to this model, release of cytochrome c from the mitochondria, and its activation of Apaf-1, are essential for all apoptosis that can be blocked by Bcl-2 (Fig. 4).

There are a large number of variations on this second model, which was based on observations that the alpha helical tertiary structure of Bcl-x resembles that of the bacterial toxin colicin and the diphtheria toxin translocation domain (Muchmore et al., 1996). In these models, Bcl-2 and Bcl-x, and their pro-apoptotic cousins Bax and Bak, act as pores through which ions or proteins can transit. In some variations of the model, Bax and Bak make the pore through which cytochrome c leaves the mitochondria, and Bcl-2 somehow blocks this pore. Another variation is that Bcl-2 and Bcl-x, like Bax and Bak, make channels through which ions pass, leading to changes in membrane potentials that indirectly determine whether cytochrome c is able to exit the mitochondria.

## Role of Bcl-2 Family Members in Vivo

Gene deletion studies have revealed a lot about the requirement for various Bcl-2 family members during development and for normal physiology, but

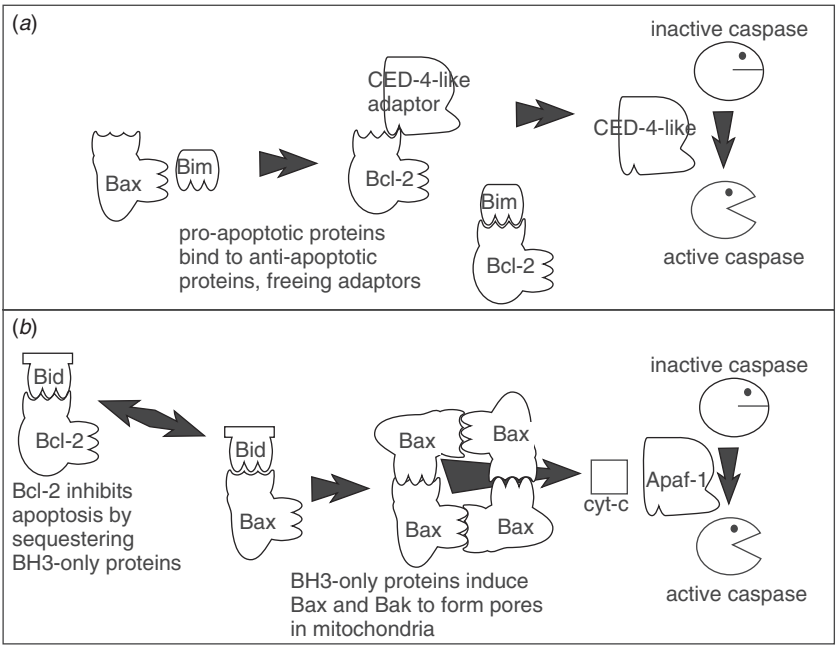


FIGURE 4. Function of Bcl-2 family proteins. There are two general models for how Bcl-2 family members work. The "adaptor inhibitor" model above is based on the genetic analysis of *C. elegans*, and suggests that unless inhibited by a BH3-only protein, antiapoptotic Bcl-2 family members prevent adaptor molecules such as Apaf-1 from activating caspases such as caspase-9, just as EGL-1 can prevent CED-9 from stopping CED-4 from activating CED-3 in the worm. The main problem with this model is that mammalian Bcl-2 does not bind to Apaf-1. The "mitochondrial pore-forming" model below suggests that the pro-apoptotic Bcl-2 family members Bax and Bak are the key players, whereas Bcl-2 and the other antiapoptotic proteins are passive "sinks" for BH3-only proteins. In this model, BH3-only proteins cause Bax and Bak to form pores in the mitochondria through which cytochrome c escapes, to then activate Apaf-1. The main problems with this model are that most BH3-only proteins do not bind to Bax or Bak, the nature of the pores formed is not clear, and it is not consistent with the genetics of cell death in the worm.

have not yet resolved which of the functional models is correct. Mice lacking Bcl-2 develop kidney disease and grey hair, and their white blood cell counts drop at an early age. Deletion of the BH3-only protein BIM in addition to Bcl-2 reverses these effects (Bouillet et al., 2001). These experiments have shown that not all of the effects of Bim are caused by antagonism of Bcl-2, and not all of the effects of Bcl-2 are caused by antagonism by Bim. In other words, antiapoptotic Bcl-2 family members such as Bcl-2 and Bcl-x can be countered by several BH3-only proteins, and each BH3-only protein may

antagonize several antiapoptotic proteins. The fact that mice heterozygous for Bim show a distinct phenotype, whereas mice heterozygous for Bcl-2, Bcl-xl, Bid, Bax, and Bak do not, suggests that Bim levels are critical, whereas levels of the other proteins are not. This means that the key point of control is likely to be at the level of certain BH3-only proteins, rather than downstream.

The resistance of cells from Bim-deleted or Bax/Bak-deleted mice to apoptosis induced by cytokine withdrawal or treatment with a variety of drugs (Wei et al., 2001) indicates that much of the regulation of apoptosis signaled via BH3-only proteins requires the presence of Bax or Bak.

## Upstream Inhibition of Caspase Activation: FLIP

Although Bcl-2 is efficient at inhibiting apoptosis induced by many agents, it is relatively poor at inhibiting apoptosis triggered by ligation of TNF receptor family members ("death receptors"), a pathway that uses the adaptor molecule FADD to activate caspase-8 (Strasser et al., 1995). This raised the possibility that other survival factors would act to control death-receptor-triggered apoptosis. Searching viral genomes for caspaselike sequences revealed several genes for proteins resembling the pro-domain of caspase-8, which were named vFLIPs (Thome et al., 1997). Once the vFLIPs were found, it was a short step to finding their cellular counterpart, now known simply as FLIP (Irmler et al., 1997).

Structurally, FLIP resembles caspase-8 and -10, consisting of two death effector domains (DEDs) and a catalytically inactive caspaselike domain. FLIP seems to work like a dominant negative form of caspase-8 (Irmler et al., 1997). By binding to FADD, FLIP can prevent caspase-8 from activating. Presumably viruses carry vFLIPs to prevent defensive apoptosis. Deletion of the genes for FLIP in mice led to embryonic lethality with abnormal heart development at day 10.5, a phenotype similar to that observed in FADD–/– and caspase-8–/– embryos. However, unlike FADD–/– and caspase-8–/– cells, FLIP–/– fibroblasts were not resistant to FasL- or TNF-induced apoptosis, suggesting FLIP may cooperate with FADD and caspase-8 during embryogenesis but antagonizes death-receptor-induced apoptosis (Yeh et al., 2000).

## Downstream Inhibition of Activated Caspases: p35 and CrmA

Just as caspases can be controlled by regulating their activation, they can also be controlled by inhibitors that bind to them after they have become active. Many viruses carry genes for upstream inhibitors of apoptosis, such as

vFLIPs or Bcl-2 homologues, so that they can keep the cell alive long enough for them to replicate (Tschopp et al., 1998). Historically, it was the study of viral antiapoptotic proteins that led to the identification of the downstream regulators of apoptosis. The first to be identified was CrmA, an inhibitor of caspase-1 and caspase-8 encoded by cowpox virus (Ray et al., 1992). By binding to caspase-8, CrmA can inhibit apoptosis triggered via TNF receptor family members (Zhou et al., 1997). Structurally, CrmA belongs to the serpin family of protease inhibitors.

The gene-encoding baculoviral protein p35 was identified by Lois Miller and colleagues as a gene deleted in a so-called annihilator strain of virus—one that induced a massive apoptotic response in insect cells which greatly reduced virus production (Clem et al., 1991). Restoring the p35 gene prevented this defensive apoptotic response, allowing time for much greater viral replication. Baculoviral p35 is able to inhibit caspases from a variety of different organisms, but no cellular homologues have been identified.

## Downstream Inhibition of Activated Caspases: The IAPs

The inhibitor of apoptosis (IAP) proteins were also identified by Miller and colleagues as baculoviral proteins that were not related to p35, but nevertheless could still complement loss of p35 in the "annihilator" strain of baculovirus (Crook et al., 1993). The ability of baculoviral IAPs to inhibit apoptosis in mammalian cells showed that they interacted with conserved components of the apoptotic mechanism, and also raised the possibility of the existence of cellular IAP homologues (Hawkins et al., 1996). Such homologues were indeed identified in insects and vertebrates by searching sequence databases for genes resembling the baculoviral IAPs (Liston et al., 1996; Uren et al., 1996). Cellular IAPs were also identified by more elegant methods, in insects in a search for mutants that modified cell death in the eyes of transgenic flies (Hay et al., 1995), and in mammalian cells as proteins in a complex associated with TNF receptor 2 (Rothe et al., 1995).

All IAPs bear one or more copies of a zinc-binding domain termed a baculoviral IAP repeat, or BIR (Hinds et al., 1999). Most also bear a second zinc-binding domain termed a RING finger that is thought to be involved in protein ubiquitination (Yang et al., 2000), but most of the functional activity of IAPs resides in the BIRs. Most IAPs have been shown to be able to inhibit apoptosis, but it appears that a subset, namely, Survivin and its homologues, are involved in cell division but not apoptosis (reviewed in Silke and Vaux, 2001).

Mammals have genes for about 10 BIR-bearing proteins, but most work has been done on XIAP, which has three BIRs and a RING finger.

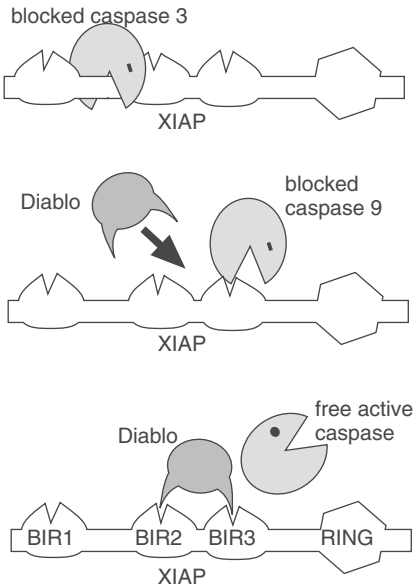


FIGURE 5. Model of XIAP function. XIAP bears three BIRs and a RING finger motif. The linker N-terminal to BIR2 can bind deeply into the catalytic site of active caspase-3 and caspase-7, inhibiting them. A pocket in the core of BIR3 of XIAP can bind to the N-terminus of processed caspase-9. Binding of the processed N-termini of Diablo/Smac or HtrA2/Omi into the pockets in the cores of BIR3 or BIR2 can displace caspases, allowing apoptosis to proceed.

## IAP Function

IAPs inhibit apoptosis by directly binding to activated caspases (Deveraux and Reed, 1999), but they can interact with the caspases in two different ways, either via a groove in the core of a BIR domain or via residues just before the BIR domain (Fesik and Shi, 2001). For example, the primary interaction between XIAP and active caspase-3 involves binding of the regions just upstream of BIR2 deep into the caspase's catalytic site, whereas the primary interaction with caspase-9 involves binding of the processed N terminus of the caspase into the core of BIR3 (Fig. 5).

## IAP Antagonists

A genetic screen in *Drosophila* for mutants in which developmental cell death was abnormal led to the identification of a number of small, pro-apoptotic proteins termed Reaper, Grim, and HID (Abrams, 1999). Subsequently, another closely linked gene was identified that encodes a fourth member of this group termed Sickle (Christich et al., 2002; Srinivasula et al., 2002; Wing

et al., 2002). All these proteins are small and their similarity is restricted to their first few amino acid residues. Fly embryos bearing the so-called H99 deletion, which removes Reaper, Grim, and HID, exhibit practically no apoptosis, demonstrating that these pro-apoptotic proteins are essential for developmental cell death.

Another genetic screen, this time for modifiers of transgenically overexpressed Reaper, yielded DIAP1, one of the *Drosophila* IAPs (Hay et al., 1995). This experiment showed that Reaper and DIAP1 worked in the same apoptotic pathway, DIAP1 as an inhibitor, and Reaper as an inducer. Subsequent experiments showed that Reaper binds directly to DIAP1 to relieve its suppression of caspase activity, and this interaction occurs mainly via the amino terminus of Reaper, the part that resembles those of HID, Grim, and Sickle.

Thus, in *Drosophila,* caspase activity can be controlled by IAPs, and the IAPs themselves are controlled by Reaper, Grim, HID, and Sickle. Because mammals also have IAPs, it was assumed they would also have IAP antagonists, but searches for mammalian proteins resembling Reaper, Grim, and HID were not productive. However, using a different approach, namely, isolating XIAP-binding proteins from mammalian cell extracts, was successful, and to date it has yielded two mammalian pro-apoptotic IAP antagonists, Diablo/Smac (Verhagen et al., 2000; Du et al., 2000) and HtrA2/Omi (Hegde et al., 2002; Martins et al., 2002; Suzuki et al., 2001; Verhagen et al., 2002).

Unlike the *Drosophila* IAP antagonists, Diablo/Smac and HtrA2/Omi are mitochondrial proteins. After synthesis, they are targeted to the mitochondrial intermembrane space by N-terminal sequences that are then removed, generating new N-termini that resemble those of the insect pro-apoptotic proteins Grim, Reaper, HID, and Sickle.

In healthy cells Diablo/Smac and HtrA2/Omi remain in the intermembrane space where they are unable to interact with IAPs, which are in the cytosol. However, if a cell receives certain apoptotic signals, proteins such as cytochrome c, Diablo/Smac, and HtrA2/Omi are released from the mitochondria. Once in the cytosol, they can bind to IAPs such as XIAP. If they are sufficiently abundant, they can compete caspase-9 away from XIAP, and caspase-9 can then process caspase-3 (Ekert et al., 2001).

## Role of IAPs in Vivo

In *Drosophila*, deletion of DIAP1 results in massive cell death and embryonic lethality (Wang et al., 1999). Partial loss of function, such as in the mutation to DIAP1 (originally known as *Thread*), causes less severe effects, with death of cells of the aristae, giving threadlike antennae. In mammals, by way of contrast, deletion of IAP genes has resulted in very minor phenotypes (Harlin et al., 2001; Holcik et al., 2000). This means either there are enough other IAPs to take over their roles (redundancy), or in mammals IAPs do not serve important functions.

By antagonizing IAPs, the *Drosophila* proteins Reaper, Grim, HID, and Sickle play essential roles in regulating apoptosis during development and in response to DNA damage. For example, *Reaper* gene expression is, in part, controlled by the *Drosophila* homologue of p53 (Brodsky et al., 2000), and HID protein can be regulated by phosphorylation by MAP kinase signaling (Bergmann et al., 1998). By comparison, Diablo/Smac-deleted mice (Okada et al., 2002) have very minor phenotype. Once again, this may reflect redundancy, or perhaps the fact that the BH3-only/Bcl-2 pathway is more important in vertebrates, whereas in insects the IAP/IAP antagonist pathway has assumed greater importance.

## Conclusion

Most of the regulation of apoptosis is achieved via the modulation of the levels or activity of survival factors. These survival factors are, in turn, controlled by inhibitory signal transduction pathways such that the cell death effector mechanisms, and the decision for a cell to survive or kill itself, can be influenced by a huge number of inputs. The importance of the roles played by survival factors is demonstrated by what happens when their genes are mutated, leading to either an increase or decrease in their activity. Overactivity of Bcl-2 caused by translocations in B cells results in the cancer follicular lymphoma. Translocations of cIAP2 are found in MALT lymphomas.

Identification of survival factors may provide targets for new anticancer pharmaceuticals. For example, a number of approaches are being investigated to decrease Bcl-2 expression by antisense oligonucleotides, or to antagonize Bcl-2 activity by agents that mimic the BH3 alpha helix. Similarly, it may be possible to counter IAP activity by drugs designed to act like the N-termini of Diablo/Smac and HtrA2/Omi.

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CHAPTER

12

# CASPASE-INDEPENDENT AND AUTOPHAGIC PROGRAMMED CELL DEATH

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The occurrence of cell death under a variety of physiological and pathological conditions in multicellular organisms has been documented many times during the past 150 years (Vogt, 1842; Fleming, 1885; Clarke and Clarke, 1995). In 1871 Virchow described the diversity of cell death as "necrosis" and "necrobiosis" (Virchow, 1871). Subsequently, numerous reports described cell death that occurs during metamorphosis of invertebrates and lower vertebrates and the development of mammals; in adults, cell loss may ensue according to physiological demands as well as after various kinds of damage by chemicals, injury, radiation, hypoxia, and so on (for a review, see Kerr et al., 1972; Wyllie et al., 1980; Beaulaton and Lockshin, 1982; Schulte-Hermann et al., 2000; Bratton and Cohen, 2001; Hickman, 2002). In developmental biology cell death mainly was considered a "programmed" event (Glücksmann, 1951; Lockshin and Williams, 1964; Lockshin et al., 2000). On the other hand, in toxicology and pathology cell death traditionally was viewed as a passive, degenerative phenomenon occurring after severe damage of tissues (for a review, see Bursch et al., 1992). It was not before the early 1970s when Farber et al.—based on a characteristic morphology of cell

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death and its requirement for protein synthesis—suggested the occurrence of a "suicide" type of cell death in liver, intestine, and other organs after treatment with cytotoxic anticancer drugs (Farber et al., 1971). The widespread occurrence and biological relevance of programmed cell death were also advocated by Kerr, Wyllie, and Currie, who in 1972 proposed a morphologically based classification of cell death into two broad categories. According to this proposal, the term "necrosis," which commonly was used to describe all types of cell death, was redefined and restricted to events caused by violent environmental perturbation leading to collapse of internal homeostasis. The new term "apoptosis" (now often and in a broader sense called programmed cell death) was coined to describe the orchestrated self-destruction of a cell (Kerr et al., 1972; Wyllie et al., 1980; Kerr, 2002). Apoptosis gained considerable credit when modern techniques provided insights into its molecular pathways. Thus, during the last decade tremendous gains in knowledge concerning the molecular events of signaling, preparation, and execution of apoptosis have been achieved (for a review, see Chang and Yang, 2000; Lockshin et al., 2000; Nicholson, 2000; Salvesen, 2001; Bratton and Cohen, 2001; Kaufmann and Hengartner, 2001; Leist and Jäättelä, 2001; Coleman and Olson, 2002; Gozani et al., 2002; Igney and Krammer, 2002; Nicotera, 2002; Martin, 2002; Mathiasen and Jäättelä, 2002).

However, a large body of morphological and biochemical evidence indicates that programmed cell death (PCD) is not confined to "classical" apoptosis, but that cells use different pathways for active self-destruction (for a review, see Clarke, 1990; Schwartz et al., 1993; Zakeri et al., 1995; Trump et al., 1997; Bursch 2001). Morphologically, three types of cell death have been discriminated: Type I is most likely identical to apoptosis (condensation and fragmentation prominent). Type II is characterized by a prominent formation of autophagic vacuoles ("autophagic cell death"). Type III is described as occurring through disintegration of cells into fragments without involvement of the lysosomal system and without marked condensation (Schweichel and Merker, 1973; Clarke, 1990). As for the molecular mechanisms underlying these different PCD morphologies, those of apoptosis are best characterized (for a review, see Chang and Yang, 2000; Lockshin et al., 2000; Hengartner, 2000; Bratton and Cohen, 2001; Leist and Jäättelä, 2001; Coleman and Olson, 2002; Mathiasen and Jäättelä, 2002). Briefly, apoptosis can be triggered by a broad range of physiological and nonphysiological signals ranging from ligation of plasma-membrane death receptors by cytokines/hormones to DNA damage by genotoxic chemicals. Mitochondria constitute a major site for integration of diverse pro-apoptotic pathways, but accumulating evidence suggests that the endoplasmic reticulum (Rao et al., 2002), lysosomes (Salvesen, 2001; Turk and Salvesen, 2002), and the trans-Golgi network (Mancini et al., 2000) play important roles as well. Thus, each organelle possesses sensors that detect specific alterations, locally activates signal transduction pathways, and emits signals which ensure interorganellar cross-talk (Ferri and Kroemer, 2001). Among the pro- and antiapoptotic

molecules bringing about the cells' suicide, a family of proteases denoted caspases plays a prominent role. In fact, their discovery provided the first evolutionarily conserved molecular machinery for initiation and final execution of the apoptotic program; caspases are responsible for most of the stereotypic morphological features of apoptotic cells (Hengartner, 2000; Chang and Yang, 2000; Coleman and Olson, 2002). The model of "caspase-dependent" apoptosis turned out to be an extremely important paradigm, but is not universal as demonstrated by the existence of caspase-independent forms of PCD (for a review, see Borner and Monney, 1999; Leist and Jäättelä, 2001; Mathiasen and Jäättelä, 2002; Nicotera, 2002). For instance, caspase-coding sequences are absent from many nonmammalian species, but nevertheless, these organisms may undergo PCD (for a review, see Fröhlich and Madeo, 2000; Wyllie and Golstein, 2001). Several authors have recently pointed out that the introduction of caspases during evolution may reflect a decisive refinement of the ancient caspase-independent death programs including autophagic PCD (for a review, see Aravind et al., 2001; Leist and Jäättelä, 2001; Wyllie and Golstein, 2001; Candé et al., 2002). Only a few studies have addressed the molecular mechanisms steering autophagic PCD in mammalian cells. Thus, evidence for the involvement of the RAS-signaling pathway has been provided by Kuchino's group (Chi et al., 1999; Kitanaka and Kuchino, 1999; Kitanaka et al., 2002). Furthermore, Kimchi and colleagues have demonstrated that DAP-kinases may control initiating steps in autophagic PCD (Inbal et al., 2002); in both instances, autophagic PCD ensues independent of caspases. However, autophagic PCD cannot generally be attributed to the "caspase-independent PCD category" as revealed by studies of insect metamorphosis (Lee and Baehrecke, 2001).

The present chapter reviews the role of autophagy in PCD. In addition to a comparative view of the morphological and functional features of autophagic PCD and those of apoptosis, an attempt is made to identify links between the autophagocytosis control in general and those molecular events that specifically may affect the life–death decision of cells. Finally, it is tempting to speculate that the diversity in PCD morphologies might reflect "caspase-functional" or "caspase-nonfunctional" pathways, and therefore, the relation of autophagic PCD to caspase-independent mechanisms of initiation and execution of cell death will be addressed.

## Morphological Diversity of PCD

### Apoptosis

Apoptosis originally was defined on the basis of a specific pattern of morphological changes in the dying cell (Kerr et al., 1972; Wyllie et al., 1980; Kerr, 2002): condensation of cytoplasm, in solid tissues separation from neighboring cells, condensation of chromatin at the nuclear membrane to sharply

delineated masses, and cell fragmentation into apoptotic bodies (Fig. 1a and b). In highly condensed dead cells or cell fragments, organelles are still intact as shown by electron microscopy; cellular membranes are well preserved and, consequently, cell contents are not liberated. In vivo, apoptotic bodies are rapidly phagocytosed and degraded by neighboring cells. In fact, apoptotic cells display "eat me signals" at their surface accommodating their recognition and rapid uptake by adjacent vital cells, and thus prevent inflammation and secondary tissue damage (Kerr et al., 1972; Wyllie et al., 1980; Savill and Fadok, 2000; Fadok and Chimini, 2001). No evidence for lysosomal or autophagic events in apoptotic cells in vivo was noted in early morphological and histochemical studies (Kerrr et al., 1972; Wyllie et al., 1980; Bursch et al., 1985). Rather, degradation of apoptotic bodies ensues as the final step of phagocytosis by vital cells (heterophagy) (Kerr et al., 1972; Wyllie et al., 1980; Bursch et al., 1985; Savill and Fadok, 2000; Fadok and Chimini, 2001).

Occurrence of Apoptosis. The occurrence of apoptosis cannot be narrowed down to certain biological conditions. Rather, apoptosis is a widespread phenomenon in the living world and plays important roles in states of health and disease (Kerr et al., 1972; Wyllie et al., 1980; Bursch et al., 1992; Krammer, 2000; Nicholson, 2000; Hickman, 2002). Nevertheless, it seems that cells consisting of relatively small amounts of cytoplasm such as nonsecretory cells enter the apoptotic pathway more readily than large, secretory cells that frequently were found to undergo autophagic PCD (see below; Zakeri et al., 1995).

## Autophagic Cell Death

Autophagic cell death, at the electron microscopic level, is characterized by the degradation of cytoplasmic components, resulting in progressive loss of electron density; the descriptions of autophagic cell death consistently include the fact that the degradation of cytoplasmic components precedes nuclear collapse (for details, see below and Fig. 1c–f). However, denoting cell death as "autophagic/Type II PCD" needs a cautionary note. A review of the literature reveals an inconsistent use of terms to describe cell death associated with autophagocytosis as it includes necrosis, nonapoptotic types of cell death, apoptosis/Type I PCD, autophagic cell death/Type II PCD, and others (for a review, see Bursch, 2001). Relatively little is known of the molecular events underlying the initiation and execution of autophagic cell death. Therefore, here an electron microscopic demonstration of autophagic vacuoles (AVs) in dying cells is taken as *conditio sine qua non* to denote cell death as autophagic/Type II PCD. Autophagosomes in the first steps of the pathway (autophagic sequestration) are separated from the cytoplasm by a double membrane followed by vacuolation of the intercisternal space and

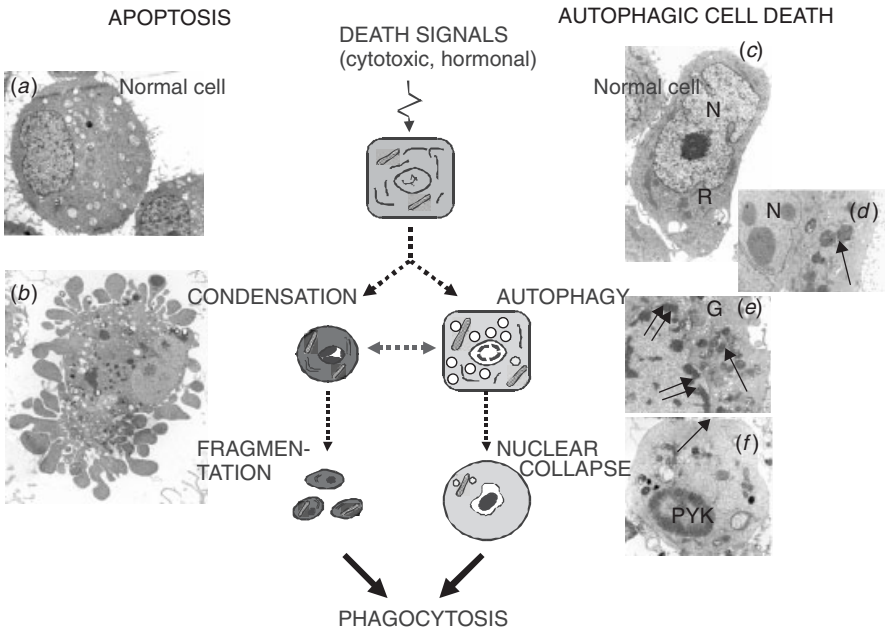


FIGURE 1. Development and patterns of cell death. *Scheme for apoptosis:* condensation of cytoplasm and of chromatin at the nuclear membrane to sharply delineated masses (often like crescents) followed by cell fragmentation into apoptotic bodies. Phagocytosis (in vivo) and heterophagic degradation. Note: According to the original description, autophagy/lysosomes do not play a distinct role early in apoptosis. Ultrastructural features of apoptosis: lung carcinoma cells (A549) (a) control and (b) fragmentation of apoptotic cell (cisplatin 24 hours, 5µg/mL) × 9,000. *Scheme for autophagic cell death*: Autophagy: formation of autophagic vacuoles (AVs; scheme: open circles) and degradation of cytoplasmic constituents. Nuclear collapse (as observed in MCF-7 cells): pyknosis, single pyknotic mass in the center of the nucleus, nuclear envelope still intact, cytoplasm amorphous with few clusters of AVs and mitochondria. Note: Autophagocytosis with apoptoticlike DNA condensation/fragmentation may also occur. Phagocytosis (in vivo) and final degradation. Ultrastructural features of autophagic PCD: MCF- 7 cultures after TAM treatment (c–f). (c) control, day 7; electron translucent vacuoles (↑), intact nucleus (N). The plasma membrane exhibits extended areas with villi; the cytoplasm typically shows multiple polyribosomes (R). (e) $10^{-6}M$ TAM, day 7; the nucleus appears normal (N); in the cytoplasm numerous AVs (↑) are visible. (f) TAM $10^{-6}M$, day 7; ribbons of condensed chromatin are detached from the nuclear envelope (↑↑). (g) TAM $10^{-6}M$, day 7; rounded cell, surface characterized by loss of microvilli. The condensed chromatin is detached from the nuclear envelope and concentrated in the center of the nucleus (PYK); the nuclear envelope appears intact (↑↑). In the amorphous cytoplasm polyribosomes are not visible; mitochondria and AVs are clustered at the cell poles (↑). Numerous AVs (↑) and the prominent Golgi regions (G) of the cytoplasm. For references, see text.

loss of inside membrane. Typical inclusions of the enveloped cytoplasmic portions comprise mixed contents like curled membranes and organelles gradually undergoing degradation (Fig. 1d–f; Seglen et al., 1996; Klionsky and Emr, 2000). In addition to electron microscopy, histo- and biochemical criteria indicating the role of the autophagosomal-lysosomal compartment can be taken into account as reviewed in detail elsewhere (Bursch, 2001). It should be emphasized that referring to the morphological/histochemical features does not imply a causative relationship between autophagocytosis and eventual manifestation of a cell's suicide; this will require either an established functional link between these phenomena and/or elucidation of specifically related molecular events. Finally, in cultured cells cytoplasmic vacuolization—which might be mistaken as autophagic vacuoles—is widely observed; however, based on their electron translucent appearance, this type of vacuole can be discriminated from autophagic vacuoles (for a review, see Henics and Wheatley, 1999).

Occurrence of Autophagic PCD. Autophagic cell death appears to be a phylogenetically old phenomenon as it has been observed in the slime mold *Dictyostelium discoideum* and in the nematode *C. elegans* (Cornillon et al., 1994; Olie et al., 1998; Hall et al., 1997). It is important during insect metamorphosis, which is one of the most extreme biological conditions of tissue remodeling; here cells of ecto-, endo- and mesodermal origin are affected (for a review, see Beaulaton and Lockshin, 1982; Clarke, 1990; Zakeri et al., 1995; Bursch, 2001). Likewise, in vertebrate development, autophagic cell death appears to be a prominent feature. It is associated with organ morphogenesis as exemplified by the shaping of extremities, cavity formation in intestine, and regression of sexual anlagen (for a review, see Beaulaton and Lockshin, 1982; Clarke, 1990; Zakeri et al., 1995; Bursch, 2001). Autophagic cell death also is reported to occur in adult insects and vertebrates including humans; it is often associated with the elimination of (large secretory) cells during adjustment of sexual organs and ancillary tissues to seasonal reproduction. As for pathophysiology, autophagic cell death has been associated with experimental and human neurological diseases, with cell injury after cytotoxic drug treatment, but also during spontaneous regression of human tumors (Kitanaka et al., 2002; for a review, see Bursch, 2001).

Taken together, autophagic PCD predominantly appears to be activated when the developmental program or (in adulthood) homeostatic mechanisms demand massive cell elimination; in all cases, the bulk of cytoplasm is degraded by autophagy before nuclear collapse ensues. In instances of cell injury, damaged organelles or membranes may be transferred into the autophagic pathway, serving as a protective response at the subcellular scale, and in the instance of the cell becoming overwhelmed, elimination of the whole cell may result. Thus, these functional features of autophagic cell death are in line with the general function of autophagy, namely, being the

major inducible pathway for degradation of cytoplasmic components including whole organelles (Blommaart et al., 1997; Klionsky and Emr, 2000). It should be noted, however, that autophagic cell death and apoptosis are not mutually exclusive phenomena. Thus, both types of cell death can occur simultaneously in tissues, but also subsequently as governed by the developmental program. Moreover, individual dying cells may exhibit both apoptotic and autophagic features ("mixed type") (for a review, see Beaulaton and Lockshin, 1982; Zakeri et al., 1995; Bursch, 2001). This morphological phenomenon now can be traced back—at least to some extent—to molecular events reflecting the "coexistence" of different suicide programs (or their high degree of plasticity) within a cell (see the section entitled "Caspase-dependent versus Caspase-independent Suicide Programs").

Remarkably, the mode of cell death does not necessarily affect the efficient clearance of cell residues from the body through phagocytosis. Thus, in vivo, autophagic PCD has been found to be completed by heterophagy (for a review, see Beaulaton and Lockshin, 1982; Clarke, 1990; Bursch, 2001). Obviously, dying cells in general display surface signals to facilitate their phagocytosis, the expression of which constitutes an integral part of the overall PCD signaling (Savill and Fadok, 2000; Fadok and Chimini, 2001).

## Molecular Diversity of Programmed Cell Death

### Apoptosis

In the last decade, tremendous progress has been made in understanding the molecular biology of apoptosis, and the reader is referred to a number of recent reviews (Chang and Yang, 2000; Lockshin et al., 2000; Nicholson, 2000; Salvesen, 2001; Bratton and Cohen, 2001; Kaufmann and Hengartner, 2001; Leist and Jäättelä, 2001; Coleman and Olson, 2002; Gozani et al., 2002; Igney and Krammer, 2002; Nicotera, 2002; Martin, 2002; Mathiasen and Jäättelä, 2002). Here, only those molecular features of apoptosis necessary for identifying the differences from autophagic PCD and the role of caspases are briefly addressed.

Caspase-dependent versus Caspase-independent Suicide Programs: A Molecular Switch for Different Cell Death Morphologies? Caspases (cysteinyl-aspartic-proteases) belong to a large family of highly conserved proteins that have been found in hydra, insects, nematodes, and mammals (Alnemri, 1997; Aravind et al., 2001). More than a dozen caspases have been identified in humans; about two-thirds of these constitute a set of sequentially acting "initiator" and "executioner" caspases mediating diverse pro-apoptotic signals down to the final coordinated self-destruction of the cell (for a review, see Thornberry and Labzenik, 1998;

Chang and Yang, 2000; Hengartner, 2000; Krammer, 2000; Ferri and Kroemer, 2001; Coleman and Olson, 2002; Köhler et al., 2002).

Activation of caspase-cascades may ensue through a number of pathways; the best-studied are the following two: (1) "extrinsic pathway," a receptor-mediated death signaling ("death receptor") that ultimately triggers caspase-8 as exemplified by the interaction of the CD95- or TNF-receptor with its ligands (Fig. 2). (2) "Intrinsic pathway," a release of a set of molecules such as cytochrome c and APAF-1 from mitochondria in response to intracellular death signals such as oxidative stress or DNA damage; cytochrome c and APAF-1 form a complex responsible for the activation of caspase-9 (apoptosome, Fig. 2). This pathway is subjected to the control of pro- and antiapoptotic members of the bcl-2 family (for a review, see Thornberry and Labzenik, 1998; Lockshin et al., 2000; Nicholson, 2000; Bratton and

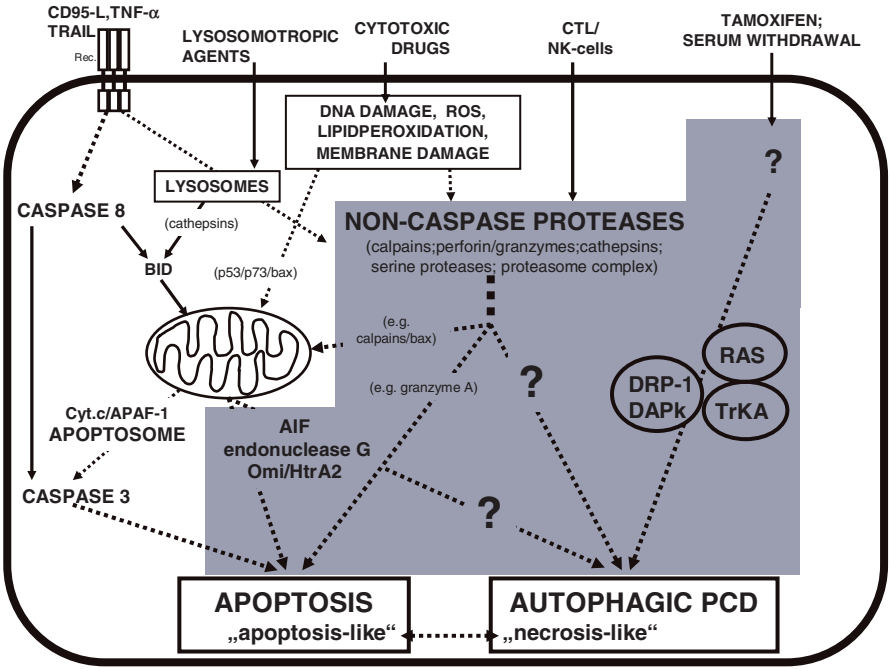


FIGURE 2. Signaling in apoptosis and autophagic PCD in mammalian cells (simplified). The figure is fully explained in the text. Lysosomotropic agents include α-tocopheryl succinate (Neuzil et al., 1999); 9-acetoxy-2,7,12,17-tetrakis-(β-methoxyethyl)-porphycene (ATMPn; Fickweiler et al., 1999); O-methyl-serine dodecyl-amide hydrochloride (MSDH; Li et al., 2000); 5,8-dihydroxy-1,4-naphthoquinone (Roberg et al., 1999); chlorin e6 triacetoxymethylester (CAME; Kessel et al., 2000); polyamine oxidase inhibitor MDL-72,527 (Dai et al., 1999); imidazo-acridinone C1311 (Burger et al., 1999); retinol (Fröhlich and Wahl, 1999). For other references, see the text. Events considered to be caspase-independent are highlighted (grid pattern).

Cohen, 2001; Kaufmann and Hengartner, 2001; Gozani et al., 2002). Both pathways are considered to join at the level of caspase-3, which is the common substrate for caspase-8 and -9, and thereby trigger the final execution of apoptosis and its characteristic morphological manifestation. The extrinsic and intrinsic pathway may communicate upstream of caspase-3 through caspase-8-mediated BID cleavage at Arg59; truncated BID targets mitochondria and stimulates release of mitochondrial pro-apoptotic molecules (for a review, see Krammer, 2000; Ferri and Kroemer, 2001; Mathiasen and Jäättelä, 2002). Further caspase activators acting downstream of mitochondria comprise Smac/Diablo and Omi/HtrA2 (both inhibit IAP-proteins) (Ferri and Kroemer, 2001; Hegde et al., 2002; Ravagnan et al., 2002). In the context of this review, it should be noted that lysosomal cathepsins may cleave BID at Arg65, and thus, a diversity of relatively nonspecific signals such as photodamage or lysosomotropic agents may be transduced to the specific enzyme cascades that trigger the apoptosis program (Fig. 2).

Caspases have been considered central executioners of the apoptotic pathway. Indeed, eliminating caspase activity, either through mutation or the small pharmacological inhibitors, slows down or even prevents apoptosis (for a review, see Chang and Yang, 2000; Hengartner, 2000; Leist and Jäättelä, 2001; Coleman and Oslon, 2002; Mathiasen and Jäättelä, 2002). However, cell suicide may also ensue in a caspase-independent fashion. In general, denoting apoptosis/PCD as belonging to the caspase-independent category mostly is based on at least one of three criteria: (1) PCD may progress in spite of the presence of caspase inhibitors. (2) PCD occurs in spite of genomic/functional knockout of a given caspase. (3) PCD is not associated with detectable caspase activation, for example, as demonstrated by the lack of specific caspase-cleavage products. However, classification of PCD as caspase-independent deserves some cautionary notes: (1) The specificity of the caspase inhibitor used may be confining. (2) An inherent cognitive problem results from the fact that *only known* phenomena (caspases) can be considered. (3) Caspase inhibition may result in the delay of PCD rather than in the rescue (survival) of cells. Thus, proof of clonogenicity should be considered essential to ascertain cell rescue and survival.

Caspase-independent apoptosis/PCD came into focus in apoptosis research not more than a few years ago, and we are currently witnessing a rapid accumulation of reports on this phenomenon; therefore, the examples addressed here are far from being complete. PCD independent of known caspases have been reported to occur in response to *drug exposure*, as in all of the following examples, among many others: in liver cancer cells treated with camptothecin (Roberts et al., 1999); mammary carcinoma cells treated with vitamin D compounds (Mathiasen et al., 1999); carcinoma cells of breast, colon, prostate, and liver; as well as glioblastoma upon Hsp70 depletion (Nylandsted et al., 2000); nonsmall lung cancer cells (NCI-H460) upon exposure to paclitaxel (Huisman et al., 2002); and human pancreatic cancer cells expressing peroxisome proliferator-activated receptor (PPAR)-γ upon

treatment with PPAR-γ agonists (Eibl et al., 2001). Furthermore, in human mammary carcinoma cells (MCF-7, MCF-7/AdrR, T47D, and SKBr3), sigma-2 *receptor agonists* have been found to induce cell death involving a p53- and caspase-independent apoptotic pathway, which is considered to be distinct from mechanisms used by some DNA-damaging, antineoplastic agents and other apoptotic stimuli (Crawford and Bowen, 2002). Cells may commit caspase-independent suicide also in response to *viral or bacterial infection* of cells. For instance, fibroblasts upon exposure to adenoviral E4orf (Lavoie et al., 1998) as well as glioma cells consequently upon exposure to an adenovirus encoding chimeric tumor suppressor 1 (Ad-CTS1) and in synergy with irradiation or CD95-ligation (Naumann et al., 2001). Productive HIV-1 infection of primary CD4+ T cells induces mitochondrial membrane permeabilization, leading to caspase-independent cell death (Petit et al., 2002). Macrophages infected with the bacterium *Chlamydia* die independent of caspases (Perfettini et al., 2002). Furthermore, caspase-independent forms of PCD have been observed in neurodegenerative and lymphatic *diseases*. Thus, the caspase-derived C-terminal fragment of beta-amyloid precursor (betaAPP) induces caspase-independent toxicity in TSM1 neurons and potentiates the pathogenic betaAPP maturation pathway by increasing selectively a beta42 species in wild-type betaAPP-expressing human cells (Dumanchin-Njock et al., 2001). Complement-mediated cell death induced by rituximab in B-cell lymphoproliferative disorders is mediated in vitro by a caspase-independent mechanism involving the generation of reactive oxygen species (Bellosillo et al., 2001). In summary, these examples demonstrate that the activation of caspase-independent PCD pathways apparently cannot be attributed to distinct biological conditions. Rather, it appears that the broad spectrum of extrinsic and intrinsic death stimuli known to activate caspase-dependent apoptosis may also trigger alternative suicide programs.

Caspase-dependent and caspase-independent pathways may coexist in the same cell and may even be coactivated. For instance, in lung carcinoma (NSCLC) cells, where the caspase-dependent pathway is less efficient, the triggering of an AIF-mediated caspase-independent mechanism circumvents the resistance of these cells to treatment (Joseph et al., 2002). Furthermore, cladribine (2-chloro-2′-deoxyadenosine) has been reported to induce apoptosis in human leukemia cells by caspase-dependent and caspase-independent pathways acting on mitochondria (Marzo et al., 2001); caspase activity is involved in, but is dispensable for, early motoneuron death in the chick embryo cervical spinal cord (Yaginuma et al., 2001). Likewise, cell suicide pathways in peripheral blood lymphocytes include caspase-independent and caspase-dependent events as revealed by studies on their response to chemotherapeutic agents (Stahnke et al., 2001). Notably, a protein may possess a bifunctional role by being involved in caspase-dependent as well as caspase-independent cascades. Thus, apoptosis signal-regulating kinase 1 (Ask1) possesses a caspase-independent killing function

that is independent of its kinase activity and may be activated by interaction with Daxx. In the physiological situation, such an activity is induced as a consequence of the translocation of Daxx from the nucleus to the cytoplasm, a condition that occurs following activation of the death receptor Fas (Charette et al., 2001). Mature Omi can induce apoptosis in human cells in a caspase-independent manner through its protease activity and in a caspase-dependent manner via its ability to disrupt caspase–IAP interaction (Hegde et al., 2002). Likewise, all death induced by the basic fibroblast growth factor (bFGF) is mediated through a caspase-dependent and p53-independent cell death receptor pathway (Westwood et al., 2002).

Caspases bring about most of the morphologically visible changes characteristic of apoptotic cell death, namely, cell shrinkage, degradation of cytoskeleton, plasma membrane blebbing, chromatin condensation, and DNA fragmentation (for a review, see Chang and Yang, 2000; Hengartner, 2000; Coleman and Olson, 2002). Cytoskeletal proteins and their regulators cleaved by caspases (mainly caspase-3, but also -6, -7, and -8) comprise actin, gelsolin, Gas2, fodrin, beta II spectrin, filamin, cadherins, catenins, keratins, vimentin, tau, FAK, and $p130^{Cas}$ (for a review, see Chang and Yang, 2000; Coleman and Olson, 2002). Lamins, the intermediate filament scaffold proteins of the nuclear envelope, are cleaved by caspase-6, leading to nuclear fragmentation in the final phase of apoptosis (for a review, see Chang and Yang, 2000; see also the section entitled "Link between Cytoplasmic (autophagic) Degradation and Final Nuclear Collapse"). In addition to morphological changes, dynamic rearrangements of the actin cytoskeleton are also central in phagocytosis (for a review, see Coleman and Olson, 2002). Nevertheless, caspase-independent cell death may share morphological and biochemical features with "classical" apoptosis. The morphology of caspase-independent modes of cell death have been described as ranging from "apoptosislike" to "necrosislike (autophagy)" as recently suggested by Mathiasen and Jäättelä (2002):

> *apoptosis-like: chromatin condensation to lumpy masses that are less compact than in apoptosis, display of phagocytosis recognition molecules, any degree and combination of other apoptotic features such as cytoplasmic shrinkage, chromatin condensation to crescent masses can be found; necrosis-like: absence of chromatin condensation or, at best, with chromatin clustering to loose speckles, varying degree of apoptosis-like changes including phosphatidylserine exposure; aborted apoptosis and type 2 physiological cell death fall in this category.*

For instance, caspase-independent cell death of alveolar macrophages dying upon exposure to cigarette smoke resembles apoptosis with respect to chromatin condensation, cell shrinkage, and mitochondrial cytochrome c release; visible nuclear condensation was not associated with chromatin fragmentation (Aoshiba et al., 2001). Macrophages infected with *Chlamydia*

undergo apoptosis in terms of morphological and biochemical features including condensation of nuclei and DNA fragmentation, but broad-spectrum caspase inhibitors failed to prevent cell death (Perfettini et al., 2002). Caspase-independent pathways that may result in a necrosis phenotype are exemplified by c-myc-Bin1-mediated cell death (Elliott et al., 2000). Likewise, TNF-induced death of fibrosarcoma cells (L929) exhibits a necrosis phenotype that is caspase-independent and does not involve DNA fragmentation; it requires increased production of reactive oxygen species (ROS) in the mitochondria and protein kinase A-mediated phoshorylation of glyoxalase I (Van Herreweghe et al., 2002). In prostate tumors treated with a combination of cisplatin and vitamin C plus K3, cell death exhibiting overlapping features of apoptosis and necrosis has been reported (the authors exclude secondary necrosis); this phenomenon was denoted "autoschizis" (Buc Calderon et al., 2002).

In a few cases, the apoptotic and nonapoptotic morphological features of the same cell can be dissected into caspase-dependent and caspase-independent events. For instance, sympathetic neurons treated with colchicine activate caspase-dependent steps (as revealed by zVAD-caspase inhibition) that appear responsible for mitochondrial swelling and nuclear fragmentation, whereas caspase-independent events affect formation of cytoplasmic vesicles; the cytoplasmic vacuolization cannot be prevented by caspase inhibitors and may eventually result in necrosis (Mitsui et al., 2001). Studies by Sperandio et al. (2000) showed that unoccupied insulinlike growth factor receptor I (IGFIR-IC) can mediate death of fibroblasts. The dying cells lack chromatin condensation and DNA cleavage; lack of caspase activation was demonstrated by the failure of the caspase inhibitors zVAD.fmk, BAF, p35, XIAP, and Bcl-$_{XL}$ to prevent IGFIR-IC-induced cell death (Sperandio et al., 2000). The cytoplasm exhibited strong vacuolization, although not of the autophagic type (see below; Sperandio et al., 2000). Paradoxically, recruitment of caspase-9—but none of the other caspases—by IGFIR appears to trigger this nonapoptotic manifestation of cell death. Sperandio et al. (2000) denoted this type of cell "paraptosis." Notably, this PCD type is not simply a result of the lack of apoptosis machinery as bax can trigger the "classical" apoptosis pathway. Likewise, studies by Chi et al. (1999) have shown that cells may switch between apoptotic (caspase-dependent) and nonapoptotic (caspase-independent necroticlike/autophagy) suicide programs depending on the cell death stimulus. These phenomena will be discussed in some more detail in the subsequent section on autophagic PCD.

Taken together, in a substantial number of cases, the morphology of caspase-independent cell death exhibits massive deviation from the classical apoptotic morphology. However, prominent morphological features of apoptosis, namely, cell shrinkage and chromatin condensation [note: not (oligo)nucleosomal DNA fragmentation] may also ensue in a caspase-

independent fashion. Obviously, the activation of caspase-independent cell death pathways does not necessarily result in shutting down cell shrinkage. Thus, caspases appear to constitute a major but not the sole determinant for the manifestation of different PCD morphologies; it seems likely that other proteases may functionally replace (known) caspases. From a teleological point of view, this seems to be an advantage for the organism: (1) A cell would be equipped with two distinct, but interchangeable, sets of enzymes to commit suicide. (2) Both pathways include condensation of dying cells as it may facilitate their phagocytosis. In this respect, it is tempting to speculate that autophagic cell death—as will be outlined below in greater detail—might reflect an additional cell death strategy, namely, not relying on precise cleavage of a limited set of crucial proteins but removal of bulk cytoplasmic constituents prior to final removal through phagocytosis. Depending on the contribution of caspase-functional and nonfunctional pathways to the cell's suicide, its morphological manifestation may result in transitional stages as described above. Furthermore, as outlined by Chang and Yang (2000), the possibility of uncoupling morphological features in apoptosis should also caution investigators to identify clearly the morphological and biochemical criteria used to measure apoptosis.

A number of noncaspase proteases have been implicated in PCD, acting either upstream or downstream of mitochondria (Fig. 2): *Perforin/granzymes* involved in CTL- and NK-cell-triggered PCD (Waterhouse and Trapini, 2002), and lysosomal *cathepsins* involved in TNF- and TRAIL-mediated PCD pathways, cell death upon exposure to detergents, to ROS generated by oxidants, as well as to lysosomotropic and DNA damaging agents (for a review, see Salvesen, 2001; Uchiyama, 2001; Bursch, 2001; Turk et al., 2002). *Granzyme A* activates an endoplasmic reticulum-associated caspase-independent nuclease to induce single-stranded DNA nicks (Beresford et al., 2001). The concerted action of endoplasmic reticulum, *calpains*, and Bax may also constitute a "caspase-alternative" suicide pathway upon exposure to differentiation-inducing agents or in response to misfolded proteins (Rao et al., 2002). Likewise, calpains may participate in the early phases of radiation-induced apoptosis, upstream of the caspases (Waterhouse et al., 1998) as well as in apoptosislike death induced by vitamin D compounds in breast cancer cells (Mathiasen et al., 2002). Mitochondria may release pro-apoptotic but caspase-independent effectors such as *apoptosis-inducing factor* (AIF), *endonuclease G*, as well as *Omi/HtrA2*, which possess serine protease activity (Candé et al., 2002; van Loo et al., 2002; Ravagnan et al., 2002; Lazebnik et al., 2002). Notably, Omi/HtrA2—in addition to its inhibition of IAPs—acts in cell rounding and shrinkage. DNA fragmentation/nuclear condensation implicates the activity of *AP24* (Wright et al., 1997). It should be also noted that exposure of "eat me signals," namely, phosphatidylserine on the outer cell membrane, implicates the action of calpains (Leist et al., 1998) or cathepsin B (Castino et al., 2002; Foghsgaard et al., 2001). The implication of caspase-

independent mechanisms in phagocytosis recognition can be seen in line with current views on the evolution of cell death programs, namely, that the introduction of caspases reflects a refinement of ancient caspase-independent death programs including auto- and heterophagy of dying cells (see below).

Finally, for the sake of completeness, it should be noted that caspase inhibition may also cause cell death. Thus, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD) or t-butyloxycarbonyl-Asp-fluoromethylketone (Boc-D) have been found to induce the death of lipopolysaccharide (LPS)-activated macrophages and RAW 264.7 cells with apoptotic features, suggesting that LPS+zVAD-induced apoptosis of macrophages is independent from the known proapoptotic caspases and the involvement of unidentified zVAD-sensitive molecule(s) (Kim et al., 2001). Likewise, broad-spectrum caspase inhibition augmented cell death in TNFα-stimulated neutrophils (Liu et al., 2002). In this context, it should be noted that caspase function is not restricted to cell death phenomena (for a review, see Chang and Yang, 2000; Hengartner, 2000).

## Autophagic PCD

Prior to discussion of the relation of autophagy to PCD in detail, it should be remembered that autophagocytosis constitutes the major inducible pathway for degradation of cytoplasmic components including whole organelles that does not necessarily result in the death of cells. It ensues through a sequence of events that are highly conserved from yeast to humans, including sequestration of cytoplasmic constituents, formation and maturation of autophagosomes, their fusion with lysosomes to give rise to autophagic vacuoles, and the final degradation of cytoplasmic material (Blommaart et al., 1997; Klionsky and Emr, 2000). By autophagocytosis cells may adapt to environmental changes such as nutrient deprivation, damage of subcellular structures including membranes, and whole organelles (Bloommart et al., 1997; Klionsky and Emr, 2000). Thus, the question arises whether autophagy might be just a side effect of the stress imposed on the cells. Or does a functional link exist between autophagocytosis and execution of the (final) death program? If so, what are the underlying molecular mechanisms? Which are the key pathways mediating external signals to autophagocytosis and/or cell death? To answer some of these questions, current knowledge on autophagy was applied in studies on nonapoptotic cell death/PCD in MCF-7 cells.

### Human Mammary Carcinoma (MCF-7) Cells as a Model for Autophagic PCD.

For almost three decades, human mammary carcinoma (MCF-7) cells have been used as a biological test system in drug development, namely, to select drugs with a strong antiproliferative potency for

treatment of human mammary tumors (for a review, see England and Jordan, 1997; Wakeling et al., 2001). More recently, we and others have used MCF-7 cells as a model to study the *antisurvival* effect of antiestrogens such as tamoxifen, ICI 164384, and toremifene (Bardon et al., 1987; Wärri et al., 1993; Otto et al., 1996; Gompel et al., 2000; Bursch et al., 1996, 2000). Thus, tamoxifen at high doses ($10^{-5}M$) causes lysis (necrosis) of almost all cells within 24 hours that cannot be prevented by estradiol (Bursch et al., 1996). The cytotoxic action of tamoxifen may result from perturbations in membrane fluidity (Wiseman, 1994), formation of reactive oxygen species (Tuner et al., 1991), and DNA damage by DNA adducts or chromosomal aberrations, which have been found to occur in kidney and liver (Han and Liehr, 1992; Sargent et al., 1994). On the other hand, lower concentrations of tamoxifen ($10^{-6}M$ and below) induced a gradual appearance of cell death that started to occur 3 days after treatment. This type of cell death is considered to be a receptor-mediated, active cell suicide because it can be inhibited by estradiol (Bursch et al., 1996). This "mitogen rescue" is considered to be characteristic of an active or programmed mode of death and is often used to functionally discriminate PCD from necrosis, which—according to this view—would be prevented only by removal of the noxious agent (for a review, see Bursch et al., 1992; Leist and Jäättelä, 2001). Closer electron microscopic studies revealed that the active self-destruction of MCF-7 cells after tamoxifen belongs to the autophagic type of PCD; representative morphological features are shown in Figure 1c–f. In MCF-7 cells upon TAM treatment, the first changes visible at the electron microscope level comprise formation of autophagic vacuoles (AV), which gradually degrade cytoplasmic structures (cf. Fig. 1d and e). Notably, in cells exhibiting a highly condensed nucleus, structures required for protein synthesis such as polyribosomes, ER, and Golgi have disappeared, whereas a few clusters of intact mitochondria persist in close vicinity to AVs and the nuclear envelope (Fig. 1f). The electron microscopic studies were confirmed and extended by histochemical studies with monodansylcadaverine (MDC), which has been described to selectively accumulate in autophagic vacuoles (Biederbick et al., 1995; Munafo and Colombo, 2001). MDC was used to visualize AVs in MCF-7 cells and to compare the kinetics of AV formation with those of nuclear condensation at the light (fluorescence) microscopic level. Clearly, AV formation preceded nuclear collapse, which reflects the irreversible stage of cell death (Bursch et al., 1996, 2000). As will be outlined subsequently, this experimental model has been successfully used to tackle a number of questions concerning the functional and molecular features of autophagic PCD.

Functional Link between Autophagocytosis and Cell Death. A functional link between autophagocytosis and cell suicide was suggested by a number of inhibition experiments with 3-methyladenine (3-MA), wortmannin, and LY294002 (Bursch et al., 1996; Chi et al., 1999; Kitanaka and

Kuchino, 1999; Jia et al., 1997; Petiot et al., 2000). Thus, 3-MA, an inhibitor of the sequestration of cytoplasmic components (Seglen and Jordan, 1982; Sandvig and van Deurs, 1992), has been found to prevent both the formation of autophagic vacuoles and the eventual cell death (indicated by nuclear destruction) in a variety of different cell types, including tamoxifen-treated human mammary carcinoma cells (MCF-7; Bursch et al., 1996), gastric and glioma cells overexpressing Ras (Kitanaka and Kuchino, 1999), TNF-α-treated human T-lymphoblastic leukemic cells (Jia et al., 1997), neuronal cells upon serum withdrawal or treatment with arabinoside (Xue et al., 1999), kidney cell lines treated with bacterial toxins such as ricin, abrin, Shiga toxin, and diphtheria toxin (Sandvig and van Deurs, 1992). Likewise, our studies on the "mitogen rescue" of MCF-7 cells with estradiol revealed that estradiol not only prevents nuclear destruction, it also inhibits the preceding formation of autophagic vacuoles.

At the molecular level, recent studies suggested the involvement specifically of the *class III* PI3-K product PtdIns(3)P in sequestration: Formation of PtdIns(3)P as well as of autophagosomes was found to be inhibited not only by 3-MA, but also by wortmannin and LY294002 (Petiot et al., 2000). Importantly, it was pointed out by Tolkovsky et al. that although 3-MA has been described to specifically block the sequestration step, its effects are not exclusively limited to the autophagic process (Tolkovsky et al., 2002). Thus, 3-MA was found to inhibit the phosphorylation of JNK and p38 kinases in NGF-deprived neurons (both may be involved in apoptosis signaling; Xue et al., 1999) and to attenuate mitochondrial permeability transition pore opening. Nevertheless, although 3-MA may affect additional pathways, studies with autophagy inhibitors other than 3-MA, namely, estradiol, wortmannin, and LY294002, strongly support a functional link between the formation of autophagic vacuoles and PCD pathway(s).

Fate of the Cytoskeleton in Autophagic PCD. In *apoptosis*, the cell's preparatory as well as execution steps include depolymerization or caspase-driven cleavage of actin, cytokeratins, lamins, and other cytoskeletal proteins, resulting in the typical final shape of apoptotic cells (see the earlier section "Caspase-dependent versus Caspase-independent Suicide Programs"; for a review, read Chang and Yang, 2000; Hengartner, 2000; Coleman and Olson, 2002). On the other hand, all steps of autophagocytosis are known to depend on certain cytoskeletal elements (for a review, see Blommaart et al., 1997; Klionsky and Emr, 2000). For instance, intermediate filaments (cytokeratin and vimentin) are necessary for sequestration of cytoplasmic structures (for a review, see Blommaart et al., 1997; Klionsky and Emr, 2000). Furthermore, all stages including the final degradation of cytoplasmic material in AVs are ATP-dependent (for a review, see Blommaart et al., 1997; Klionsky and Emr, 2000). Therefore, we investigated whether cleavage of the cytoskeleton as described for apoptosis would, or would not, occur

during *autophagic cell death*. The fate of cytoskeletal elements was closely followed during autophagic cell death in individual MCF-7 cells after TAM by immunocytochemistry; in addition, the protein pattern was analyzed by biochemical means (Bursch et al., 1996, 2000). Indeed, the cytoskeleton was found to be redistributed but largely preserved, even in cells exhibiting nuclear condensation/fragmentation, that is, the irreversible stage of cell death (Bursch et al., 1996). A pronounced fragmentation of the cytokeratin was not detected before MCF-7 cells detached from the substrate, which is a very late stage of cell death in MCF-7 cells and probably reflects secondary necrosis (Bursch et al., 2000). Remarkably, the vast majority (about 85%) of MCF-7 cells still contained F-actin when the nucleus was already condensed (Bursch et al., 2000). Polymerization of G- to F-actin is an *ATP-dependent* process and, therefore, F-actin is a sensitive indicator of the metabolic state of a cell. In support of this notion, electron microscopy and rhodamine 123 staining revealed that even at late stages of the death process, autophagic vacuoles were associated with clusters of structurally and functionally intact mitochondria although most of the cytoplasm appeared amorphous (Fig. 1f; Bursch et al., 1996, 2000). It appears likely that ATP synthesis is maintained during the progress of autophagic cell death at a level required for the completion of autophagocytosis. Moreover, *transglutaminase*, an enzyme cross-linking proteins and subcellular structures, is activated in apoptotic hepatocytes (Bursch et al., 1992), but not in tamoxifen-induced PCD of MCF-7 cells. Thus, the preservation of the cytoskeleton during autophagic death of MCF-7 cells agrees with current concepts on the cytoskeleton's function in autophagy.

Link between Cytoplasmic (Autophagic) Degradation and Final Nuclear Collapse. This topic constitutes the most enigmatic part of the autophagic cell death sequence. In apoptosis, many of the essential players have been identified. Briefly, the typical morphology of apoptotic nuclei, namely, condensation of chromatin to crescent masses abutting to the nuclear membrane, results from a specific sequence of cleavage events. Thus, the genomic DNA is cleaved into large chromatin domains of 50 to 300 kbp, and then in many but not all cell types to oligonucleosomes (for a review, see Zhang and Xu, 2002). All but the earliest large-domain cleavage events depend on caspases: The caspase-activated DNAse (CAD/DFF-40), normally sequestered in the cytoplasm by the chaperone ICAD/DFF-45, is released and translocated to the nucleus once the chaperone has been degraded by caspase-3, finally resulting in the oligonucleosomal cleavage pattern. Furthermore, the nuclear envelope becomes discontinuous, and the lamin polymer that normally underlies the nuclear membrane is broken down by proteolysis (Goldberg et al., 1999). What about the nuclear destruction during autophagic PCD? Cell death induced in caspase-3-deficient MCF-7 cells by TNF-α as well as staurosporine was not associated with low

molecular weight (LMW), that is, (oligo)nucleosomal DNA fragmentation, cell shrinkage, and blebbing (Jänicke et al., 1998). We have confirmed and extended these observations by showing that caspase-3-deficient MCF-7 cells exhibit high molecular weight (HMW) DNA fragmentation into 50 to 300 kbp (Bursch et al., 1996). These observations agree well with others showing that HMW-DNA fragmentation is brought about by caspase-independent mechanism(s). Alternative noncaspase proteases conceivably involved in completion cell suicide may include cathepsins (Roberts et al., 1999), calpains (Doerfler et al., 2000), serine proteases (Wright et al., 1997), granzymes (Johnson, 2000), and/or the proteasome complex (Jones et al., 1995; Johnson, 2000). A nonapoptotic type of cell death was identified in insect tissues characterized by strong expression of the polyubiquitin gene and of the multicatalytic proteinase (proteasome; Schwartz et al., 1993). Ubiquitin binds to cellular proteins to label them for proteolytic degradation by the proteasome protease. In our studies MCF-7 cells treated with tamoxifen showed neither induction of ubiquitin mRNA expression nor synthesis of proteasome protein above control level. However, preliminary results of our own obtained with high throughput two-dimensional gel electrophoresis of subcellular fractions suggest translocation of proteasome subunits ($\alpha$, $\delta$, $\zeta$, $\tau$) from the cytoplasm to the nucleus. Interestingly, Arnoult et al. reported that cytoplasmic extracts from dying *Dictyostelium* cells were found to trigger the breakdown of isolated mammalian and *Dictyostelium* nuclei in a cell-free system (Arnoult et al., 2001). DNA fragmentation was prevented by a polyclonal antibody specific for *Dictyostelium discoideum* apoptosis-inducing factor (DdAIF), and therefore the authors suggested that DdAIF is involved in DNA degradation during *Dictyostelium* cell death. A further candidate is endonuclease G, which has been found to be involved in CAD-independent DNA fragmentation during cell death pathways in which truncated Bid is generated (van Loo et al., 2002).

Exciting new insights into molecular events regulating cytoplasmic and nuclear destruction have been provided by studies on developmental cell death in *Drosophila* (Lee and Baehrecke, 2001). Thus, salivary gland cell death during development of *Drosophila* has been found to include autophagocytosis. At the molecular level, the gene E93 was reported to be sufficient to trigger cell death and, based on gain-of-function studies, E93 is considered to be necessary for autophagy (Lee and Baehrecke, 2001). The downstream effector of E93 includes *crq* (croquemort) for pro-autophagic signaling, a gene that is required for phagocytosis during normal embryonic development (Franc et al., 1999). Importantly, other downstream E93 effectors, namely, *rpr*, *hid*, and *grim*, are considered to be essential for nuclear apoptotic responses such as DNA fragmentation (Lee and Baehrecke, 2001). Taken together, these data suggest that in *Drosophila* the E93 gene may constitute a key regulator driving a concerted cytoplasmic and nuclear breakdown during autophagic PCD.

Molecular Control and Signal Transduction for Autophagic PCD. One of the first molecular links between autophagy and programmed cell death in mammalian cells has been provided by studies on the RAS-signaling pathway: expression of oncogenically mutated Ras gene in human glioma and gastric cancer cell lines induced cell death associated with autophagocytosis (Kitanaka and Kuchino, 1999; Chi et al., 1999; Kitanaka et al., 2002). The nuclei remained relatively well preserved and were negative for TUNEL staining, thus matching the features of autophagic PCD (cf. Table 1; Kitanaka and Kuchino, 1999; Chi et al., 1999; Kitanaka et al., 2002). Furthermore, Ras-induced cell death occurred in the absence of caspase activation, it did not require wt-p53 activity, and it was not inhibited by the antiapoptotic Bcl-2 protein (Kitanaka and Kuchino, 1999; Chi et al., 1999; Kitanaka et al., 2002). These features of Ras-induced cell death as demonstrated in experimental systems were recently confirmed and extended by clinical observations on spontaneous regressing neuroblastoma in humans: Cell death was found to be associated with increased Ras expression, but lack of capase-3 activation and DNA fragmentation (Kitanaka et al., 2002). Notably, the functional effector machinery for the execution of apoptosis could be activated in the Ras-transformed cells by TNF-α, demonstrating that the manifestation of autophagic cell death does not simply reflect defective apoptosis (Chi et al., 1999). Rather, cells apparently switch among different suicide pathways depending on the external death signal. Likewise, MCF-7 may enter the autophagic, caspase-independent PCD pathway upon antiestrogen treatment, whereas TNF-α/TRAIL-induced cell death of MCF-7 cells was found to involve activation of the initiator caspase-8 at the apex of a caspase cascade including cleavage cytoskeletal proteins (Fig. 2, Table 1; MacFarlane et al., 2000).

Of note is that cell death induced by the oncogenic Ras was dependent on the activity of phosphatidylinositol-3-kinases (PI3-K), a physiological downstream effector of Ras (Chi et al., 1999). In turn, PI3-kinases downstream effectors comprise the mTOR/p70S6-kinase pathway, which is considered to be the master switch between catabolism and anabolism of cells; mTOR may also exert a plethora of functions in various pathways of programmed cell death (Blommaart et al., 1997; Klionsky and Emr, 2000; Raught et al., 2001; Castedo et al., 2002). The hypophosphorylated p70S6-kinase promotes detachment of ribosomes from endoplasmic reticulum, presumably one of the initial molecular events in the sequestration step of autophagy (for a review, see Blommaart et al., 1997; Klionsky and Emr, 2000). mTOR also might turn out to link recent observations on some of the yeast Apg-genes (autophagy-defective genes) to cell death. To date, 14 Apg-genes are known to act in a conjugation cascade controlling the initiation and execution of autophagy; notably, these molecular processes have been found to be highly conserved from yeast to humans as reviewed elsewhere (Blommaart et al., 1997; Mizushima et al., 1998; Klionsky and Emr, 2000). In the context of pro-

TABLE 1. Differences and commonalities between apoptotic (type I) and autophagic (type II) PCD

| | AUTOPHAGIC PCD | APOPTOTIC PCD | |
|---|---|---|---|
| | MCF-7 / TAM | MCF-7 / TNFα | Jurkat cells / CD95L<br>Hepatocytes / TGFβ1 |
| Autophagic vacuoles | + | – | – (+)* |
| Stress response<br>(hsp 90 translocation) | + | n.d. | + |
| Protein synthesis | | | |
| (auto)phagosomes: annexin VI, grp78 | + | | – |
| lysosomes: cathepsin B, D | – | n.d. | n.d. |
| Cytoskeletal protein breakdown | | | |
| intermediate filaments, fodrin[1,2], actin[2],<br>myosin light chain | – | + | + |
| Caspase involvement | – | + | + |
| Transglutaminase involvement | – | n.d. | + |
| Nuclear protein breakdown | | | |
| SAF-A[2], SATB1[2], p40, lamin B[2,3] | – | n.d. | + |
| SUPT6H, HA95, PWP-1 | + | | + |
| DNA fragmentation | | | |
| HMW | + | + | + |
| LMW | – (±) | – (±) | + |
| TUNEL (in situ) | – (±) | – (±) | + |

HMW = high molecular weight (50, 300 kbp) and LMW = low molecular weight (20 kbp and below) DNA fragmentation as revealed by PULSE-field and conventional agarose gel electrophoresis (Bursch et al. 1996). (1): calpain target; (2) caspase 3 target; (3): caspase 6 target; SAF: scaffold attachment factor A, STAB1: Special AT-rich sequence binding protein 1; p40: laminin–binding receptor precursor p40; (4–6) chromatin-associated proteins: SUPT6H: putative chromatin structure regulator; HA95: neighbor of A-kinase anchoring protein95; PWP-1: nuclear phosphoprotein similar to S. cerevisiae PWP-1. n.d.: not determined. (+)* under cell culture conditions we occasionally have observed the occurrence of autophagic vacuoles in intact as well as apoptotic cells; although autophagy in these cases appears not to be related specifically to the induction of cell death, this phenomenon should not be concealed (Bursch 2001). For further references: see text.

grammed cell death, two members of this gene family will be discussed briefly. First, human Apg5 (hApg5) was considered to be homologous to "apoptosis specific protein" (ASP; Hammond et al., 1998), and this work has been repeatedly cited as providing evidence for a molecular link between programmed cell death and autophagy (for a review, see Yung et al., 2002). However, this hypothesis can no longer be maintained. Thus, as most recently demonstrated by Tolkovsky and coworkers, the apoptosis-specific protein (ASP 45kDa) and hApg5 are unrelated proteins that share the property (along with other proteins) of interacting with c-jun polyclonal antibodies used in the earlier studies (Yung et al., 2002). Second, beclin-1 (Apg6/vps30) has been described as inducing autophagocytosis in mammalian cells; it is a bcl-2-interacting protein with structural similarity to the yeast autophagy gene apg6/vps30 (Aita et al., 1999; Liang et al., 1999). In MCF-7 cells, the autophagocytosis-promoting activity of beclin 1 was associated with the inhibition of MCF7 cell proliferation, in vitro clonogenicity and tumorigenesis in nude mice, but no evidence for an induction of cell death was observed (Liang et al., 1999). However, most recent studies on neurodegeneration in Lurcher mice provided evidence for the interaction of beclin-1 with two other proteins, namely, the mutated glutamate GluRdelta2(Lc) receptor and nPIST, a novel isoform of a PDZ domain-containing protein that binds to this receptor, and that the interaction of these proteins results in autophagic death of cerebellar Purkinje cells (Yue et al., 2002).

Most recent studies by Kimchi and coworkers revealed the death-associated protein (DAP)-kinases as important regulators for both apoptosis and autophagic PCD (Inbal et al., 2002). DAP-kinases (DAPk) are a group of $Ca^{2+}$ calmodulin-regulated serine/threonine kinases, known for a few years to be involved in a wide array of apoptotic pathways initiated by interferon-$\gamma$, TNF-$\alpha$, CD95-L, and detachment from extracellular matrix (Cohen and Kimchi, 2001). Recently, DAPk-related protein kinase (DRP-1) was isolated as a novel member of the DAP-kinase family of proteins (Shani et al., 2001). DRP-1 and DAPk have been found to possess rate-limiting functions in two distinct cytoplasmic events, namely, membrane blebbing (characteristic of apoptotic cell death) as well as extensive autophagy (typical of autophagic PCD). These two different cellular enzyme activation outcomes occurred independent of caspase activity (Inbal et al., 2002). Furthermore, expression of a dominant negative mutant of DRP-1 or of DAPk antisense mRNA reduced autophagy induced by antiestrogens, amino acid starvation, or administration of interferon-gamma (Inbal et al., 2002). Notably, these mutants did not prevent nuclear fragmentation, suggesting that DRP-1 and DAPk specifically act in signal transduction for cytoplasmic rather than nuclear degradation (Inbal et al., 2002). In line with this, immunogold staining showed that DRP-1 is localized inside the autophagic vesicles (Inbal et al., 2002). Taken together, these findings strongly suggest the direct involvement of DRP-1 in the process of autophagy.

Biogenesis of Lysosomes in Autophagic PCD. As outlined thus far, there is cumulative evidence that a set of molecules closely associated with the *initial steps* of autophagocytosis also appears to affect the life–death decision of cells. What about interactions between the control of *biogenesis of lysosomes* (Luzio et al., 2000) and their subsequent fusion with autophagosomes with that of the cell's suicide? In regressing endocrine-dependent tumors, de novo synthesis and the increased activity of lysosomal enzymes were described (Gullino, 1980). More recently, TNF-α was found to induce an autophagic type of cell death in T-lymphoblastic leukemic cells; 3-MA inhibited both the formation of autophagosomes and cell death (Jia et al., 1997). However, asparagine, which inhibits the fusion of lysosomes with autophagosomes, did not prevent TNF-α-induced cell death (Jia et al., 1997). Thus, inhibition of an event downstream of sequestration did not affect the execution of autophagic cell death, suggesting that at least in T lymphocytes the supply of lysosomes might not be a checkpoint for initiation of this type of PCD. Furthermore, increasing the lysosomal pH by monensin or $NH_4Cl$ did not protect kidney cells against ricin-induced lysis, a type of cell death exhibiting characteristic features of PCD (Sandvig and van Deurs, 1992). Notably, 3-MA has been reported to slightly increase lysosomal pH (Tolkovsky et al., 2002). However, as an increase in lysosomal pH seems not to prevent cell death, an increase in lysosomal pH as a possible cause for the protective action of 3-MA in the MCF-7 model of autophagic PCD appears unlikely (Sandvig and van Deurs, 1992). In support of this hypothesis, tamoxifen-induced autophagic cell death in MCF-7 cells was found not to be associated with an expansion of the lysosomal compartment. Thus, based on histochemical and biochemical studies, we found evidence of neither an increased rate of synthesis nor activity of lysosomal proteases (Table 1; L. Török, U. Fröhwein, C. Gerner, W. Bursch, unpublished observation). In summary, at present the interactions of lysosome biogenesis with the pathway(s) leading to autophagic cell death remain elusive. However, the current functional and molecular data suggest that the events controlling the formation of autophagosomes rather than the biogenesis of lysosomes might provide a superior regulatory link(s) between autophagocytosis and cell suicide.

## Differences and Commonalities between PCD Patways: Is Autophagic PCD Caspase-Independent?

As outlined above, apoptosis and autophagic PCD are not mutually exclusive phenomena; they may occur simultaneously in tissues. The same cell also may respond to death signals by entering either the apoptotic (Type I) or autophagic (Type II) PCD pathway as demonstrated by Kuchino and coworkers (Chi et al., 1999; Kitanaka and Kuchino, 1999; Kitanaka et al.,

2002). Studies on isolated neurons revealed that the manifestation of autophagic cell death may be controlled upstream of caspase cascades, but downstream of JNK/p38 (after NGF withdrawal) and p53 (after cytosine arabinoside; Xue et al., 1999). These studies also suggested that the same apoptotic signals which target mitochondria also activate autophagy (cf. Fig. 2, Tolkovsky et al., 2002). Once activated, autophagy may mediate caspase-independent neuronal cell death (Xue et al., 1999). The lysosomotropic agent chloroquine induced cell death with overlapping features of neuronal autophagic PCD and apoptosis, namely, concentration- and time-dependent accumulation of autophagosomes, caspase-3 activation; cell death was inhibited by 3-methyladenine, but not by Boc-Asp-FMK (BAF), a broad caspase inhibitor. Furthermore, targeted gene disruptions of p53 and bax inhibited that of bcl-x-potentiated chloroquine-induced neuron death. Caspase-9- and caspase-3-deficient neurons were not protected from chloroquine cytotoxicity (Zaidi et al., 2001). In studies with MCF-7 cell cultures, at the morphological level a subfraction of dying cells showed autophagic cell death with an apoptotic nuclear morphology (Bursch et al., 1996). At the biochemical level, some nuclear proteins are cleaved during apoptosis *and* autophagic cell death such as SUPT6H, HA95, and PWP-1; other nuclear proteins are cleaved exclusively during apoptosis (of Jurkat cells) but *not* during autophagic PCD, for example, lamin-B and the scaffold attachment factor (Franc et al., 1999; Table 1). Likewise, autophagic and apoptotic PCD seem to share the cell's stress response as indicated by translocation of heat shock protein-90 (Table 1). Recent studies of the role of DAP-kinases also revealed some commonalities between the apoptotic and autophagic death pathway (Inbal et al., 2002). Thus, the expression of both the death-associated protein kinase (DAPk) and DAPk-related protein kinase (DRP-1) was found to trigger two major cytoplasmic events: (1) membrane blebbing, which is characteristic of "classical" apoptosis, and (2) autophagy as typical of Type II PCD. In conclusion, although a few molecular pieces such as the DAP-kinases and RAS-signaling pathway in mammals as well as the E93-pathway in *Drosophila* have emerged recently, the specific molecular pattern(s) of autophagic cell death remain to be proven.

Remarkably, a number of observations suggest that the autophagic type of cell death ensues independent of caspases. Thus, neuronal cell death was induced by RAS expression (Chi et al., 1999; Kitanaka and Kuchino, 1999; Kitanaka et al., 2002), death of sympathetic cells (Xue et al., 1999), death of MCF-7 after tamoxifen (C. Gerner and U. Fröhwein, unpublished observation), or as a consequence of DAP-kinase expression (Inbal et al., 2002). However, salivary gland cell death during development of *Drosophila* has been reported to include autophagocytosis but also to require caspase activity; the role of caspases at distinct stages of autophagy is not clear (Lee and Baehrecke, 2001). Thus, autophagic PCD cannot be specifically attributed to caspase-independent pathway(s) of PCD.

What would the advantage of autophagic PCD be? It is tempting to speculate that self-digestion preceding suicide might reduce the functional load imposed on the surviving cells by phagocytosis and breakdown of huge amounts of dead cells as necessary in remodeling tissues; thereby, a rapid elimination of cells would be facilitated and help to prevent inflammatory and immunological responses. In addition, soluble molecules resulting from autophagic breakdown might be recycled by other mechanisms such as pinocytosis. Autophagy preceding cell death may also reflect a cell's adaptive response to sublethal (nonnecrotic) conditions such as nutrient/growth factor deprivation or cell damage by cytotoxic drugs, hypoxia, and so on.

## Conclusions

PCD is an essential phenomenon in normal development and adulthood of multicellular organisms. Cells use different ways for active self-destruction, with the morphology ranging from apoptosis to autophagic cell death. A functional link between autophagocytosis and a subsequent nuclear collapse (cell death) is suggested by a number of studies showing that 3-methyladenine inhibits both formation of autophagosomes and the manifestation of cell death (nuclear collapse). In mammalian systems, molecular links between autophagocytosis and eventual cell death have been provided by recent findings on DAP-kinases and Ras-signaling (including PI3-kinases). Additional—but less clear—evidence for molecular events that might be associated with a cell's choosing autophagic PCD includes mTOR/p70/S6 signaling and the autophagocytosis gene apg6/vps30 (beclin-1). In *Drosophila*, the E93 gene appears to be a key regulator, driving a concerted cytoplasmic and nuclear breakdown during autophagic PCD. However, apoptosis and autophagic cell death are not mutually exclusive phenomena; they may occur simultaneously in tissues or even conjointly in the same cell. In vivo, cell residues resulting from both processes may be cleared by heterophagy. It should be emphasized that autophagic and apoptotic PCD appears to be highly conserved during evolution as it occurs in unicellular organisms (Ameisen, 1996), in the green alga *Volvox* sp. regulating the germ-soma dichotomy (Kirk et al., 1987), in the slime mold *Dictyostelium discoideum* (Cornillon et al., 1994; Olie et al., 1998), and last but not least, in plants (Beers and McDowell, 2001). Golstein and coworkers developed the hypothesis that a single core mechanism of PCD may have developed before the postulated multiple emergences of multicellularity (Cornillon et al., 1994; Olie et al., 1998). According to this hypothesis, the phenotypic variations of PCD would result from differences in a cell's enzymatic equipment and mechanical constraints. Probably the "older" autophagic cell death pathway has been improved by caspases rendering possible more precise and more rapid cutting down of molecules essential for survival of cells. Evolutionary conservation of death pathways would

increase the organism's flexibility to respond to physiological and nonphysiological demands.

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CHAPTER

13

# THE RECOGNITION AND ENGULFMENT OF APOPTOTIC CELLS BY PHAGOCYTES

RAYMOND B. BIRGE

## ABBREVIATIONS

| | |
|---|---|
| *C. elegans* | *Caenorhabditis elegans* |
| Crk | CT10-regulator of kinase |
| DOCK180 | Downstream of Crk-binding protein |
| SH2 domain | Src homology 2 domain |
| SH3 domain | Src homology 3 domain |
| PTB domain | Protein tyrosine-binding domain |
| LRP | Low density lipid-related protein |
| ABCA1 | ATP-binding cassette transporter 1 |
| SP-A, SP-D | Surfactant protein A, D |
| SR-A (B, C, D, E, F) | Scavenger receptor class A (B–F) |
| MBL | Mannose-binding lectin |
| C1q, iC3b | Complement factors |
| CRP | C-reactive protein |
| LOX-1 | Lectin like oxidized LDL receptor-1 |
| SAP | Serum amyloid protein |
| PTX | Pentraxin |

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| | |
|---|---|
| Ig | Immunoglobulin |
| CR3, CR4 | Complement receptors 3, 4 |
| PAMP | Pathogen-associated molecular pattern |
| ACAMP | Apopototic cell-associated molecular pattern |
| TGF-β | Transforming growth factor-beta |
| IL-10 | Interleukin-10 |
| $PGE_2$ | Prostoglandin E2 |
| PS | Phosphatidylserine |
| PS-R | Phosphatidylserine receptor |
| αvβ5/αvβ3 integrin | Alpha v beta 3,5 integrin |
| MAC | Membrane attack complex |
| ICAM | Intercellular adhesion molecule-3 |
| Gas-6 | Growth arrest gene factor-6 |
| MFG-E8 | Milk fat globule EGF factor-8 |
| TSP | Thrombospondin |
| Mϕ | Macrophage |
| DC | Dendritic cell |
| RPE | Retinal pigmented epithelial cells |
| ROS | Rod outer segments |
| VN | Vitronectin |
| $Mer^{KD}$ | Mer receptor tyrosine kinase-dead |
| APC | Antigen-presenting cells |
| CTL | Cytotoxic T lymphocyte |
| MHC | Major histocompatibility complex |
| FAK | Focal adhesion kinase |
| PKC | Protein kinase C |

During evolution, the progression from unicellular autonomous organisms to socialized multicellular organisms led to a fundamental set of new biological rules with respect to how cells recognize and respond to damaged or unnecessary neighbors. Not only did such early multicellular organisms have to learn to socialize and "love thy neighbor," they also had to learn to "respect their dead." As part of this doctrine, not only did life and death decisions change meanings with respect to the survival of the organism, but once death occurred, the remaining living cells were responsible for disposing of corpses to prevent them from decaying and poisoning the tissue environment. In this capacity, cells in multicellular organisms have coevolved mechanisms to cannibalize their neighbors in a safe and efficient manner. As outlined below, the recognition and efficient clearance of apoptotic cells by phagocytes occur via evolutionarily conserved and tightly regulated pathways, whose function is essential for the homeostasis of the multicellular organism.

## Lessons From *C. elegans*

The genetics of cell death learned from studies in *C. elegans* established the notion of a centralized cell death machine, collectively referred to as the "apoptosome." In *C. elegans*, the basic machinery is encoded by three genes: *ced-3*, a pro-caspase (pro-Ced-3); *ced-4*, an Apaf-1-related procaspase activator; and *ced-9*, a Bcl-2-like cell death inhibitor (1). This machinery is inactive but poised to become activated in all somatic and germ line cells. Following a pro-apoptotic stimulus, the Ced-9 antiapoptotic function is antagonized, leading to Ced-4-dependent activation of Ced-3, and the execution of various aspects of apoptosis (2). The final result of apoptosis is not simple cell destruction and dissolution, but rather a complex network of cell interactions in which the apoptotic cell interacts with a phagocyte to initiate signals that promote rapid internalization. In the *C. elegans* model, seven genes have been identified (*ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, *ced-10*, *and ced-12*) that, when mutated, induce a phenotype in which apoptotic corpse removal or digestion is impaired (3–5). Based on the conceptual framework established for the apoptosome, it is tempting to speculate that the aforementioned engulfment genes might comprise interacting modules, such as ligand–receptor pairs, or signaling networks that internalize apoptotic cell-bound receptors, perhaps termed an "engulfosome," which is fundamentally the same in all phagocytosing cells. This teleological argument, which in certain aspects may be correct, also appears oversimplified based on the diversity of phagocytosis receptors and pathways in mammalian cells. A centralized engulfosome analogous to the apoptosome required for all engulfment pathways has yet to be observed experimentally.

## Engulfment Models in *C. elegans*

The genetic studies in *C. elegans* revealed that the seven genes involved in engulfment act in two distinct and partially redundant complementation groups. The Ced-2, Ced-5, and Ced-10 proteins are components of a Rac-GTPase signaling network that regulates cytoskeletal reorganization, cell adhesion and motility, and membrane ruffling and filopodial formation (6–8) (Fig. 1). Interestingly, mutations in *ced-2*, *ced-5*, or *ced-10* genes result not only in defects in engulfing corpses, but also in defects in distal tip cell migration, suggesting that cytoskeletal events governing phagocytosis and cell migration must be fundamentally similar (6, 9). Ced-2 is similar to the SH2/SH3 domain containing Crk II protein (10) and Ced-5 is similar to human DOCK180, which was originally cloned as a Crk-SH3-binding partner (downstream or CRK signaling) (11, 12). Ced-10 encodes the worm homolog of Rac1, a Rho-family GTPase involved in polarized cell migration (6). In addition to the Crk-binding motif, DOCK180 itself contains a centralized

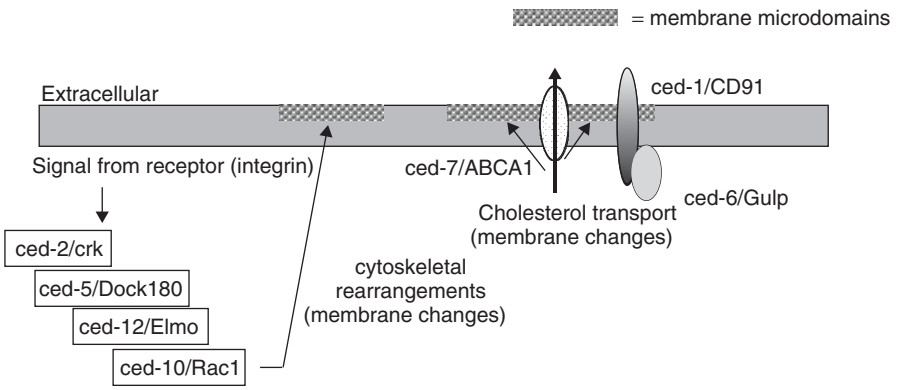


FIGURE 1. Ced-2, ced-5, ced-12, ced-10, ced-1, and ced-6 are signaling modules that mediate phagocytosis. In the phagocytosing cell, the Ced-2, -5, -10, -12 module is involved in Rac activation, a Rho-GTPase implicated in cytoskeletal and membrane reorganization. Ced-6 is a PTB-domain adapter protein that interacts with the cytoplasmic tail of Ced-1, and may be regulated by Ced-7, a lipid transport protein. Both complementation groups have effects on membrane structure and function, and as illustrated, the possibility exists that these pathways create as yet unidentified membrane microdomains (the engulfosome) required for apoptotic cell recognition (shown as hatched microdomains on the membrane).

DOCKER domain that binds Rac1, and an N-terminal SH3 domain that binds Ced-12/ELMO (13). Recent biochemical studies suggest that together the complex of DOCK180 (Ced-5)/ELMO (Ced-12) constitutes an unusual guanine nucleotide exchange protein for Rac1, resulting in GTP loading and activation (14). Hence, the Ced-2, Ced-5, Ced-10, and Ced-12 complementation group comprises an integrated network of signaling proteins that can form a molecular complex to activate Rac1 at the plasma membrane. In mammalian cells, the Crk/DOCK180/Rac1 complex is brought to the plasma membrane by the activation of $\alpha v\beta 5$ integrin, although it is not yet clear what receptors in the nematode are upstream of this module (Fig. 1). An important question for both mammalian and nematode studies will be to elucidate how universally Crk, DOCK180, and Rac1 are employed in postreceptor engulfment pathways.

The second complementation in *C. elegans* comprises the *ced-1*, *ced-6*, and *ced-7* genes. In general, the nature of interaction of the gene products is less defined compared to the GTPase pathway described above since only two of the three gene products (Ced-1 and Ced-6) exist in a functional complex. Ced-6, as well as its human homolog GULP, encodes a Shc-like adapter protein, containing an N-terminal phosphotyrosine binding (PTB) domain, a central leucine zipper that mediates protein dimerization, and a carboxyl-terminal serine/proline-rich region (15–17). Although originally Ced-6/

Gulp was identified as an "orphan adapter," it has now been clearly demonstrated that Ced-6/Gulp can bind to the cytoplasmic domain of Ced-1 (18). Ced-1 encodes a transmembrane receptor that contains atypical EGF-like repeats in the extracellular domain, a single transmembrane spanning domain, and a short cytoplasmic domain that contains a NPXY and YXXL motifs, the former of which mediates a specific interaction with the PTB domain of Ced-6 (19, 20). Employing a combination of *in silica* informatics studies as well as classic biochemical confirmation, Ravichandran and colleagues identified the mammalian homolog of Ced-1 as CD91/LRP (low density receptor-related protein), an endocytic/phagocytic receptor implicated in the phagocytosis of microbial pathogens (18). Interestingly, using a Ced-1-GFP reporter protein (where GFP was fused to the intracellular domain of Ced-1), Zhou and colleagues showed that Ced-1 becomes clustered around apoptotic cells at the plasma membrane, suggesting that it binds a specific ligand on the apoptotic cell which triggers receptor capping and internalization (19). Although Ced-6 binding to Ced-1 may trigger reorganization of the actin cytoskeleton, possibly via a Rho family GTPase, it is unlikely that Ced-1 activates the Crk-DOCK180-Rac1 switch, at least from genetic studies, since *ced-2*, *5*, or *10* do not compensate for *ced-1* or *ced-6* mutations in the nematode (21).

The third gene in this group, *ced-7* (ABCA1), encodes a protein that belongs to the large family of ATP-binding cassette proteins or ATP-hydrolyzing proteins which are associated with active membrane transport processes (22, 23). Although the function of ABC1 is not completely understood, experiments utilizing neutralizing antibodies or pharmacological inhibitors suggest that ABCA1 functions as a lipid transporter and may play a role in general phospholipid organization and topology (24). One of the most interesting aspects of Ced-7/ABCA1 biology is that the protein is required on both the phagocytic cell as well as the apoptotic cell (25). Although it is possible that Ced-7 participates in a homotypic interaction, erythrocytes isolated from ABCA1-deficient mice exhibit defects in phosphatidylserine (PS) externalization that normally result from increases in cytosolic calcium. This supports the idea that Ced-7/ABCA1, at least on the apoptotic cell, promotes PS externalization which serves as a recognition signal to the phagocyte (22, 26). Interesting, with respect to the phagocyte biology, studies by Zhou et al. showed that Ced-7 was required for Ced-1-mediated receptor clustering around the apoptotic cell (19). Therefore, an attractive idea, posited by Hengartner, suggests that Ced-7 indirectly promotes Ced-1/Ced-6 signaling by regulating specific lipid microdomains, such as Cholesterol-rich membrane domains or rafts or caveoli, or not yet defined microdomain compartments that regulate engulfment modules (27) (Fig. 1). It is also noteworthy that Crk (via binding $p130^{cas}$) induces the formation of membrane ruffles, which themselves contain membrane rafts (9). Therefore, returning to the issue of the putative engulfosome described

above, such a concept might be envisioned as a higher-order membrane structure in which central components of the engulfment machinery reside. Although speculative, such microdomains might be specialized for phagocyte/apoptotic cell recognition, as well as housing signaling molecules required for internalization (Fig. 1).

## Changes in Cell Surface Molecules Contribute to the Recognition of Apoptotic Cells by Phagocytes

The generally accepted paradigm for engulfment of apoptotic cells is that apoptotic cells display "eat me" signals which are recognized and processed by the phagocyte (28, 29). In all organisms investigated to date, the most prominent cell surface change is the exposure of PS on the outer leaflet of the plasma membrane. In mammalian cells, the phospholipids of the plasma membrane are asymmetrically distributed between the inner and outer bilayer leaflets (the extracellular leaflet membrane contains zwitterionic phospholipids, phosphatidylcholine, and sphingomyelin, and the intracellular leaflet membrane partitions aminophospholipids, phosphatidylethanolamine, and PS) (24). With respect to the function of PS in normal cells, such asymmetry is maintained by the action of at least two competing enzymes, an ATP-dependent translocase or "flipase" that actively transports PS to the inner membrane, and a calcium-dependent scramblase that functions to randomize phospholipids in the plasma membrane (24). During apoptosis, for reasons that are only beginning to be understood and that are in part cell-type-specific (30), the activities of the cytoplasmically directed translocases are inhibited, and the activity of the scramblase activated, resulting in the rapid relocalization of PS (31). In mature erythrocytes, for example, the loss of PS-translocase activity observed during apoptosis is blocked by Z-DEVD-FMK inhibitors, indicating that PS exposure may occur downstream of caspase activation (32). It is also noteworthy that Ced-7 may potentially serve as an ATP-dependent translocase which transports PS from the inner to outer membrane (33). Although the generality of ABCA1 being a PS transporter is still controversial, it is apparent that redundant pathways for PS exposure can occur, and early PS exposure on the extracellular surface is clearly a central theme with respect to the recognition of apoptotic cells by phagocytes.

However, it is important to note that such a loss in transmembrane phospholipid asymmetry and PS exposure in apoptotic cells is only a single readout of many other complex perturbations in the biophysical and biochemical properties of the plasma membrane. Indeed, there is now a great deal of evidence demonstrating the complex modification of lipids, sugar chains, and glycoproteins on the surface of the apoptotic cell that is recognized by scavenger receptors on the phagocytes (34). Although the nature of

the altered lipids and proteins and the mechanism(s) by which they are modified remain unclear, mass spectrometry and biochemical studies by Podrez et al. identified specific oxidized species of phosphatidylcholine (PC), but not nonoxidized PC, as high-affinity ligands for the type-B scavenger receptor CD36 (35, 36). Interestingly, in some cases, PS oxidation has also been implicated in both the PS externalization and recognition by macrophages, again suggesting that phospholipid oxidation is an important component of phagocyte recognition (37). Other changes in the apoptotic cell membrane include alterations in mannose- and galactose-containing glycoproteins (38), recruitment of heparin-binding sites (39), and the modifications of adhesion molecules such as ICAM-3, the latter of which is recognized by CD14 on phagocytes (40). In general, the relevant ligands on the apoptotic cell have not been well characterized. Clearly, a careful survey by mass spectrometry to identify altered glycoproteins during apoptosis, analogous to what has been performed to identify proteins in the phagosome (41), is a valid and important use of modern proteomics.

## Role of Phagocytosis in the Cell Death Program: Evidence for Bidirectional Signaling between Phagocytes and Target Cells

Although it is clear that specific "eat me" signals on the apoptotic cell trigger recognition by the phagocyte, new genetic studies in the *C. elegans* suggest that phagocytes play a much more active role in the cell death process than originally realized (42, 43). Hence, in the refined models, phagocytes are not simply bystander cells, but participate in a nonautonomous manner to the killing. While there has been a great deal of speculation about the rationale for such bidirectional signaling and how relevant it may be in vivo (44, 45), the idea is attractive because it suggests that apoptosis and phagocytosis are tightly coupled. As discussed below, persistent apoptotic corpses in the absence of engulfment proceed to a secondary necrosis, which in turn can lead to the production of pro-inflammatory cytokines and tissue inflammation, or possibly fragments of apoptotic blebs that could serve as autoantigens. In the nematode studies, the effects of the feedback circuit were most striking in partial *ced-3* loss of function mutants and not observed in mutant worms that completely lacked *ced-3*, indicating that the signals from the phagocyte positively feed back to the central apoptosome machinery, resulting in caspase activation (42, 43).

The idea that phagocytes actively participate in the process of apoptosis raises a number of potentially interesting questions. What is the nature of the signals initiated by the apoptotic cell? What are the counteractive signals contributed by the phagocyte and are they reversible? How quickly are the signals transmitted and in which cell compartments do the "committed

steps" occur? With respect to the recognition stages, these could include classic "eat me" signals, such as exposure of PS on the outer leaflet of the cell, as well as modified lipids and carbohydrates (29). In addition, Savill and colleagues advanced a conceptual argument that in addition to "eat me" signals, cells also use another mechanism in which repulsive cues that typically repel live cells and phagocytes are "disabled" (46). In the first example, during apoptosis, homophilic ligation of platelet-endothelial cell adhesion molecule 1-PECAM-1 or CD31 (a signal that would normally mediate detachment and repulsion) is inactivated, reversing the repulsive signal and promoting cell-cell tethering between the apoptotic cell with the macrophage. Perhaps it is in this context that life and death decisions between the apoptotic cell and the phagocyte are decided. Moreover, from a practical viewpoint, if the phagocyte mechanism of cell death is widespread, then extreme caution must be exhibited in interpreting apoptosis studies in vitro or without phagocytes. Typically, in vivo, apoptotic cells, with distinctive condensed and marginated nuclei, are found inside membrane-bound phagocytic vacuoles, and indeed this may be the more physiological milieu for the execution phase of apoptosis.

## Multiple Receptors in Mammalian Cells Participate in the Phagocytosis of Apoptotic Cells

The recognition of apoptotic cells by receptors on phagocytes is an exceedingly complex process in which multiple receptors simultaneously interact with multiple surface components of the apoptotic cell (Table 1). In part because the nature of the apoptotic cell membrane has not been fully appreciated, the full complement of surface proteins that recognize apoptotic cells is also not fully known. The complexity of receptor utilization in mammalian cells is clearly different from the genetic studies in *C. elegans*, where only two transmembrane proteins (Ced-1 and Ced-7) were identified. Why are so many receptors used in the recognition of apoptotic cells? The answers to these questions are beginning to emerge based on studies showing that ingestion of apoptotic cells can influence multiple differentiation and immunological outcomes in immune cells such as macrophages and dendritic cells (DCs) (47). Indeed, specialized and nonoverlapping receptor systems can permit bifurcations toward specific postreceptor signaling events as well as variations in trafficking and processing of the apoptotic cell once internalized. Clearly, the importance of receptor redundancy will be better defined as the downstream signaling of the individual receptors becomes more clearly elucidated. As outlined in Fig. 2, although not mutually exclusive, there are four general subclasses of receptors that recognize apoptotic cells. These receptors recognize (1) pattern recognition, (2) oxi-

TABLE 1. Phagocytic Receptors (left column) Involved in the Recognition and Clearance of Apoptotic Cells. The putative ligands on the apoptotic cell (middle column) and the Indication whether the ligands require soluble opsonizing factors (right column) to facilitate receptor binding are noted

| Surface Receptor | Ligand | Requires Opsonization |
|---|---|---|
| CD91/calreticulum (collectin receptors) (Ced-1) | C1q, SP-A, SP-D, MBL | Yes |
| CD14 | ICAM-3 | No |
| FCγRI, III | IgG, CRP, SAP, PTX | Yes |
| CR-3/CR-4 | C1q, iC3b | Yes |
| Scavenger receptors (SR-A, SR-B1, CD36, CD68, SREC, Lox-1 Croquemort) | OxLDL, phospholipid | No |
| C-Mer/Tyro-3 | Gas-6, protein S | Yes |
| PS-R | Phosphatidylserine (PS) | No |
| β-GPI-R | β-GPI | Yes |
| Ced-7 | Ced-7 | No |
| αvβ5/αvβ3 | MFG-E8, TSP | Yes |

dized phospholipid/LDL-like structures, (3) in the context of PS, and (4) in the context of homotypic interactions (Fig. 2).

## Subclass I: Pathogen-associated Molecular Patterns versus Apoptotic Cell-associated Molecular Patterns

An interesting analogy has been recently proposed suggesting that structures on the surface of apoptotic cells share common epitopes with structures on the surface of the microbial pathogen, the so-called pathogen-associated molecular patterns (PAMPs) (48). The basic argument is that (i) apoptotic cells, like microbial pathogens, are able to activate complement proteins and recruit acute-phase serum proteins to their surface, and (ii) once opsonized, the modified apoptotic cells are recognized by pattern recognition receptors important for the innate immune response (49–52). Such receptors include CD14 (the LPS receptor), CD91/calreticulm, complement receptors CR3 and CR4, Fc-γR1, and several of the scavenger receptors (Table 1). As part of the innate immune response, soluble opsonizing factors that include immunoglobulins and complement factors recognize PAMPs, and through direct binding coat microorganisms that gain access to the host envi-

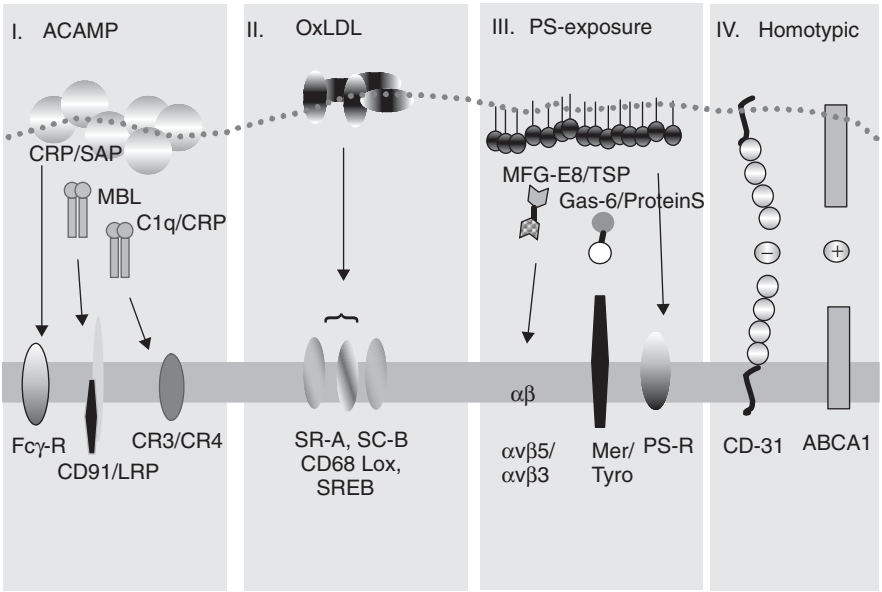


FIGURE 2. Mechanisms of apoptotic cell recognition by phagocyte receptors. Many receptors have been identified on the phagocyte membrane (see Table 1). Based on the discussion in the text, the receptors can be grouped into four subclasses based on how they recognize the apoptotic cell. These include receptors such as FC-γRI, CR3, CR4, and CD91, implicated in the innate immune response, that recognize ACAMP on the apoptotic cell in the context of complement or acute-phase proteins (panel I); scavenger receptors (SR-A, SC-B, CD68, Lox, and SREB) that recognize oxidized LDL and phospholipids on the apoptotic cells (panel II); signaling receptors, such as integrins, Mer, and PS-R, that recognize apoptotic cells within the context of PS exposure (panel III); and CD31 and Ced-7/ABCA1 that recognize apoptotic cells homotypically, either to promote engulfment (ABCA1) or promote repulsion (CD31) (panel IV).

ronment, thereby permitting recognition and engulfment by immune cells. By a mechanism that is not clearly understood, apoptotic cells also induce activation of both the classical and alternative complement pathways, resulting in the formation of intermediates that include C1q, iC3b, and mannose-binding lectin (MBL) (members of the collectin family of proteins) (52) (Fig. 2, panel 1). Once generated, these factors bind and opsonize apoptotic cells, and similar to PAMPs, direct their binding to receptors such as CR3 and CR4 (53, 54).

However, an important distinction between PAMPs and apoptotic cell-associated molecular patterns (ACAMPs) has to be drawn given the vastly different inflammatory responses mediated when phagocytes encounter pathogens versus apoptotic cells. Phagocytic engulfment of bacterial

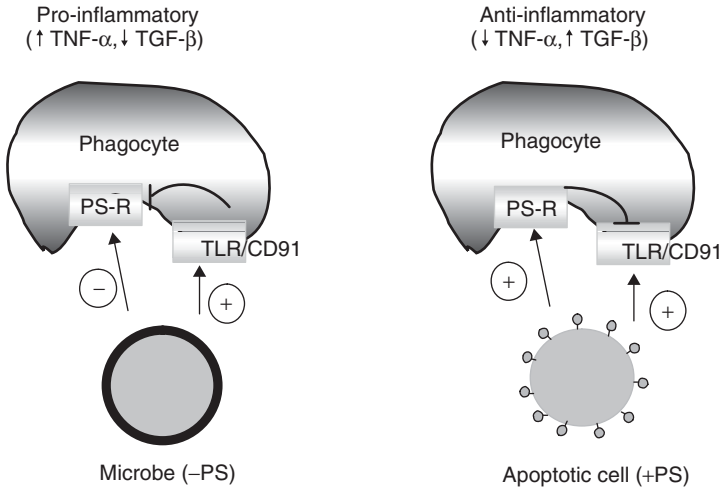


FIGURE 3. PS exposure on the apoptotic cell acts as an antiinflammatory molecular switch. As indicated in the text, both microbial pathogens and apoptotic cells utilize similar receptors for phagocytic clearance. An interesting model, by Fadok and colleagues, suggests that PS exposure on the apoptotic cell, but not on the bacterial cell wall, downmodulates the inflammatory response, and induces secretion of TGF-β and other antiinflammatory cytokines from the phagocyte.

pathogens promotes a robust pro-inflammatory response, whereas engulfment of apoptotic cells mediates secretion of antiinflammatory factors such as transforming growth factor β (TGF-β), $PGE_2$ (prostaglandin E2), and interleukin-10 (IL-10), which block inflammation (55, 56). In an attempt to resolve this conundrum, Fadok and colleagues suggest that one mechanism accounting for these distinctions is the expression of PS itself on the surface of the apoptotic cell (57, 58). These investigators suggest that the binding of PS to the PS receptor (PS-R) on the phagocyte acts as an overriding molecular switch, bypassing the inflammatory response (Fig. 3). Because of their cell walls, microbial organisms do not possess surface PS, and hence do not elicit PS-mediated antiinflammatory signaling. Necrotic cells, possibly via the leakage of cytoplasmic PS-binding proteins such as annexin V or cytosolic protease which cleave the PS-R, mask PS-R–PS interactions and thereby bypass the antiinflammatory switch. Consistent with this latter example, cystic fibrosis patients show infiltrating neutrophils that secrete elastase, which cleaves and inactivates the PS-R, resulting in downmodulation and concomitant impairment of phagocytosis in the resident alveolar macrophages (59). Clearly, an important question pertains to the molecular mechanisms of PS-mediated TGF-β upregulation, and whether other receptors, besides PS-R, participate in cytokine secretion.

### Pentraxans and Apoptotic Cell Clearance

A second but related mechanism that directs "safe" clearance of apoptotic cells is mediated by acute-phase proteins, which belong to the family pentraxin family of cyclic pentameric proteins that include C-reactive protein (CRP), serum amyloid P component (SAP), and PTX3 (60). Like components of classic and mannose-binding lectin pathways, pentraxans also bind components of the early apoptotic cell membrane (61) and direct them to the phagocyte through Fcγ receptors (62). Although the epitopes have not been completely confirmed, possible candidates include PS, PE, oxidized PC, as well as heparin and components of chromatin, such as histones and DNA, that might be exposed on the surface of the apoptotic cell (60, 63). Interestingly, both CRP and SAP potentiate the activation of the early complement pathway in the context of the apoptotic cell membrane, and promote the accumulation of C1q, C3b, and iC3b on the surface, but block the assembly of the terminal complement components and the formation of the membrane attack complex (MAC) that would result in necrotic cell lysis and inflammation (60). This is important since CRP has been shown to promote macrophage phagocytosis in a noninflammatory manner, accompanied by the secretion of TGF-β (64). These results again suggest that recognition pathways, designed for pattern recognition and the clearance of microbial pathogens, have coopted specialized pathways that are able to recognize apoptotic cells, but cleverly do so in the absence of inflammation and tissue injury.

### Subclass II: Scavenger Receptors and Apoptotic Cell Recognition

A second group of surface glycoproteins implicated in the recognition and clearance of apoptotic cells are the scavenger receptors (SRs) (Fig. 2). Scavenger receptors represent a complex multifunctional family of cell surface glycoproteins that bind a diverse array of foreign material (such as Gram negative and Gram positive material through LPS), as well as modified endogenous material including oxidized and acetylated lipoproteins (LDLs) (34). Relevant to the discussion here, SRs also recognize the modified surfaces of apoptotic cells (65–67). Although all are characterized as having highly complex extracellular recognition and extremely short intracellular domains, the intracellular domains appear to be important for cell signaling and function (68, 69). Accordingly, SRs have been grouped into six subgroups (SR-class A through SR-class F) based on the nature of their extracellular structural motifs (34). Curiously, although structurally divergent, several SRs have been shown to participate in apoptotic cell clearance, including SRA-1, SR-A2, SR-B1, CD36, SR-C1, and Lox (class E-SR) (34). Moreover, in *Drosophila* embryos, where macrophages are the predominant

cell for apoptotic cell clearance, deficiencies of the dCD36 (called Croquemort), result in low efficiency of apoptotic cell clearance, suggesting that the SR pathway, at least in some circumstances, may play a predominant role in developmental phagocytosis (70). From these observations, it is again apparent that altered self-components of the apoptotic surface, likely related to oxidized LDLs, mimic recognition components utilized as part of the innate immune response.

## Subclasses III and IV: Signaling Receptors in the Phagocytosis of Apoptotic Cells

An interesting group of receptors that recognize and internalize apoptotic cells is classified as receptors that have coopted during specialized functions in the phagocytosis of apoptotic cells. Such receptors include the αvβ5 and αvβ3 integrin receptors, the Mer receptors tyrosine kinase, and PS-R (Table 1). Since the original observation by Savill's group that αvβ3 integrin, in a complex with the type-B scavenger receptor CD36, promoted uptake of apoptotic cells by macrophages (71, 72), several subsequent studies have corroborated the findings that αvβ3, and its related receptor αvβ5, mediate engulfment in other cell types, including immature DCs (73), retinal pigmented epithelial cells (RPE) (74), and epithelial cells (7). αvβ3 and αvβ5 integrins are also critical for proliferation, cell adhesion, and migration of smooth muscle cells and endothelial cells required during angiogenesis, including pathological conditions that occur during tumor growth and metastasis (75). For cell adhesion and migration, the ligand for these integrins is an RGD motif in the extracellular matrix (ECM) molecule vitronectin (VN). VN provides a physical link between the cytoskeleton, where the force needed for contraction is generated, and the extracellular matrix. Integrin activation is closely associated with the activation of tyrosine kinases, such as FAK- and Src-family kinases, which transmit signals resulting in the PI-3 kinase and Rho-GTPases (76).

Recent studies suggest that recognition and interaction of αvβ3 and αvβ5 integrins in the context of the apoptotic cells occur not by ECM, but rather by RGD-containing soluble opsonizing molecules MFG-E8/lactadherin (77, 78) and thrombospondin (TSP) (72, 79, 80). Both MFG-E8 and TSP are secreted glycoproteins that appear to play a primary role in cell adhesion, but also because of their multidomain structure, they bridge apoptotic cells to phagocytes via αvβ3 and αvβ5 integrins. Structurally, both MFG-E8 and TSP contain aminophospholipid-binding sites that bind PS, as well as N-terminal RGD motifs which bind to αvβ3 and αvβ5 integrins (65). Nagata and colleagues not only showed that MFG-E8 stimulated engulfment of apoptotic cells in thioglycolate-stimulated macrophages, but also that mutations in the RGD domain blocked uptake and served as dominant negative

MFG-E8 mutants (78). Hence, together with previous biochemical data showing that integrins activate tyrosine kinases and mediates Rac1 activation through Crk, DOCK180, and ELMO (7), these data suggest that MFG-E8 and TSP, when bound to the apoptotic cell, probably act as RGD-containing ligands to activate integrin signaling. In macrophages, αvβ3 integrin-mediated uptake of apoptotic cells was similarly dependent on tyrosine phosphorylation (phosphotyrosine was shown to accumulate around the phagocytic cup) and inhibited by blocking Rac/Cdc42 activation (81). It is also important to note that TSP, and potentially MFG-E8, are also ligands for CD36 and can bridge αvβ3 integrin and CD36 into a functional complex (72) (Fig. 4b). With respect to the tyrosine phosphorylation and cell signaling, CD36 is interesting for two reasons. First, CD36 can associate with Src-family members such as Lyn, Fyn, and Yes (82), which can potentially mediate phosphorylation of $p130^{cas}$. Second, CD36 is predominantly associated with caveolin-rich Triton X-100 insoluble membrane rafts, which are greatly enriched in cellular signaling proteins (83). Although CD36 has been shown to recruit

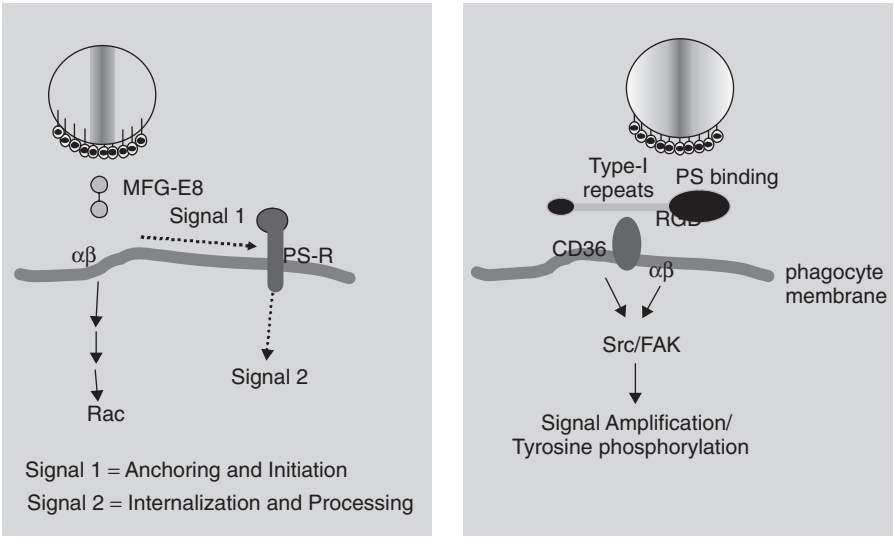


FIGURE 4. Examples of molecular cross-talk between phagocytic receptors. Panel A: the "tether and tickle" hypothesis (113). In this model, the first receptor (e.g., αvβ3 or αvβ5 integrins) serves to tether or recruit the apoptotic cell to the phagocyte membrane, whereas a second receptor (PS-R) promotes internalization. In panel B, the soluble bridging protein TSP serves to cross-link αvβ3 integrin with the CD36 scavenger receptor. Cross-linking CD36 with integrins may recruit integrins to membrane rafts and potentiate tyrosine kinase signaling.

α6β1 and α3β1 integrins into membrane rafts (84), an unexplored but important question is whether TSP or MFG-E8, indirectly through CD36, recruits αvβ3 or αvβ5 integrins into membrane rafts.

A second tyrosine phosphorylation-dependent pathway important for the clearance of apoptotic cells emerge from studies showing that macrophages expressing a kinase-dead Mer product are defective in phagocytosis (85). Moreover, mutant mice expressing $Mer^{KD}$ exhibit an accumulation of remnant apoptotic cells in their thymus and develop autoimmune illness similar to systemic lupus erythematosis (SLE) (86). Mer is also mutated in the Royal College of Surgeons (RCS) rat strain that develops retinal degeneration and shows defects in rod outer segment (ROS) phagocytosis in RPE cells, further supporting the conclusion of Mer's role in the recognition and internalization process (87). Mer (Tyro-12) is structurally related to two other receptor tyrosine kinases, Axl (Tyro-7) and Rse (Tyro-3), which together play a role in the development of several tissues, including cells of the immune system (88). Analogous to MFG-E8 and TSP, the ligand for Mer is growth-arrest-specific gene-6 (Gas-6), a multidomain opsonizing protein containing an N-terminal domain that binds PS, several EGF-like repeats, and a large C-terminal steroid-hormone-binding protein-link domain that can activate the tyrosine kinase activity of Mer (89). The ligand for Rse (Tyro-3) appears to be protein S, a vitamin-K-dependent serum protein involved in blood coagulation that is highly homologous to Gas-6 (90). Depletion of protein S from serum markedly reduces macrophage-mediated phagocytosis of apoptotic cells, suggesting the important role of these opsonizing agents in targeting apoptotic cells to the Mer-family receptors. Presently, the signaling pathway responsible for Mer/Tyro-3-mediated phagocytosis is unclear, and like αvβ3 and αvβ5, activation of Mer has been linked to several downstream signaling pathways, including activation of PI3-kinase and Rac1 (91, 92), although it is not clear whether Mer or Tyro-3 signaling converge on Crk and DOCK180.

## PS-R Signaling and Receptor Cross-talk

For both the integrin and Mer-receptor tyrosine kinase-dependent engulfment pathways described above, PS exposure on the apoptotic cell plays a pivotal role in the recruitment of opsonizing proteins, MFG-E8, TSP, Gas-6, and protein S. However, a direct PS-binding receptor has also been molecularly characterized that directs PS on the apoptotic cell to the phagocyte in the absence of bridging proteins (93, 94). Although overexpression of PS-R resulted in enhanced uptake of apoptotic cells and antibodies against PS-R block uptake, the molecular mechanisms of PS-R-mediated signaling are not well understood. Topologically, the PS-R likely acts as a Type II membrane protein and might possibly become an ectoprotein on the external surface of

the phagocyte under certain conditions. Although the PS-R is not homologous by sequence to other known proteins, it does contain a WW domain implicated in intracellular signal transduction, and potential tyrosine phosphorylation sites (93, 94). However, cell biological studies by Hoffmann and colleagues provided some compelling evidence that PS-R functions in a two-step model, by internalizing apoptotic cells that have been "tethered" to the phagocyte membrane by other receptors and mechanisms (Fig. 4a). Using a clever system in which biotinylated red cell membranes were used as "apoptotic particles" to target individual receptors, including αvβ3 integrin, αvβ5 integrin, CD14, and CD36, these investigators showed that PS-R functions as the predominant internalizing pathway, while the scavenger receptors and integrins appear to have a tethering or recruitment function (95). Although such studies require molecular validation, the studies do support the idea that different receptors have distinct functions with respect to apoptotic cell recognition and internalization, and importantly, proper uptake appears to require cross-talk among different receptors.

## Modulation of Phagocyte Function

During the engulfment phase the phagocyte must adapt to the stress invoked by the engulfment process. First and foremost, the phagocyte must protect itself from the potentially lethal cargo of the apoptotic cell, which includes caspases and other degradative enzymes. With respect to phagocyte cell survival, recent studies in macrophages suggest that the process of phagocytosis of apoptotic cells actually promote cytokine-independent survival via the activation of PI3-kinase and the Akt pathway (96). Perhaps related to this, it has also been shown in trypanosome-infected macrophages (with the protozoan *Trypanosoma cruzi*) αvβ3 integrin-mediated engulfment of apoptotic cells drives the growth and survival of the parasite (97). Although these findings might simply reflect the phagocyte's attempt to avoid toxicity, they also raise an intriguing idea that there may be metabolic or nutritional value to the phagocyte in cell corpse eating.

## Modulation of Immune Responses

Because phagocytosis generally occurs early in the apoptotic process, prior to the loss of membrane integrity, an argument has been made that phagocytosis is a passive noninflammatory event. However, as alluded to above, it is now recognized there is a much more active attempt to minimize inflammation during engulfment. For example, during engulfment many cell types, including macrophages, dendritic cells, and nonprofessional

cells, actively secrete antiinflammatory cytokines, such as transforming growth factor (TGF)-β, prostaglandin E-2 ($PGE_2$), and interleukin 10 (IL-10), and platelet-activating factor (PAF) (57). As these factors act as paracrine factors to downmodulate other pro-inflammatory factors such as TNF-α and macrophage inflammatory protein-2 (Mip-2), phagocytosis of apoptotic cells is not only antiapoptotic for the phagocyte, but also creates an anti-inflammatory milieu that permeates the surrounding tissue (98). TGF-β secretion from thioglycolate-stimulated macrophages was dependent on the ligation of PS on the apoptotic cells to the phagocyte, mediated at least in part by the PS-R (58), although the contribution of the integrin receptors, Mer-RTK, and CRP signals for antiinflammatory signaling presently cannot be ruled out.

Consistent with the idea that the rapid "safe" physiological clearance of apoptotic cells is required for the resolution of inflammation, improper handling and persistent accumulation of apoptotic cells in tissue have been linked to autoimmunity and the development of a SLE-like disease (52). For example, in humans, deficiencies in C1q lead to increased residual apoptotic bodies in the kidney and renal pathology, concomitant with the appearance of increased circulating autoantibodies against self-components (99). Similarly, mice that are deficient in CRP or SAP, or that express mutant Mer-receptors, also manifest autoimmune disease with delayed degradation of chromatin and spontaneous development of lupuslike disease (100). Presumably related to the breakdown of the nuclear envelope structure and events related to nuclear condensation during apoptosis, apoptotic cells and their membrane "blebs" are potent sources of chromatin, DNA, and nuclear antigens that can be sources of autoantigens if released into tissue. Consequently, defects in clearance, mediated by mutations or loss of activity of opsonizing factors or their receptors, would lead to secondary necrosis and the leakage of these antigens into the tissue. Recent studies by Nagata and colleagues showed that chromosomal DNA fragmentation, a hallmark of apoptosis in the dying cell, is facilitated by nucleases within the intracellular lysosomes of the phagocyte, suggesting that engulfment is required for the degradation of potential autoantigens (101). These investigators showed that apoptotic thymocytes derived from mice defective in caspase-activated DNase (CAD) were resistant to intrinsic DNA fragmentation, but could degrade DNA inside the phagocyte upon coculturing with wild-type macrophages. Similar observations have been reported in *C. elegans*, whereby the DNA of the apoptotic cells is also degraded by *nuc*-1 in the phagocyte, and in *Drosophila melanogaster*, defects in CAD-like nucleases lead to DNA accumulation and the activation of an anti-DNA immune response (102). Again, these studies suggest that inflammation resulting from the mishandling of apoptotic cells can lead to autoimmune disease, and that the etiology of lupuslike disease may reside in faulty phagocytosis mechanisms.

## Specialized Postengulfment Pathways; Phagocytosis in Dendritic Cells and Antigen Presentation

Recently, there has been considerably interest in elucidatcing how apoptotic cells, once engulfed and processed, affect the function of immature DCs (48). Whereas both macrophages and DCs are professional "scavengers" capable of engulfing apoptotic cells and pathogens, immature DCs, in contrast, are potent antigen-presenting cells (APCs) to naive T cells and are uniquely specialized to initiate T-cell immunity by activating a class I-specific CTL response (47) (Fig. 5). DC activation is governed by the maturation of immature DCs, which involves the downmodulation of endocytic and phagocytic receptors, and the concomitant upregulation of MHC molecules and costimulatory molecules on the surface of the DCs, which are capable of interaction with T cells via the T-cell receptor (103). Similar to the case of macrophages, engulfment of apoptotic cells by immature DCs under physiological conditions induces TGF-β and antiinflammatory cytokines, and fails to induce DC maturation (104, 105). However, under certain conditions that are not well understood, engulfment of apoptotic cells can induce DC maturation and the presentation of apoptotic cell antigens, via a process called "cross-presentation." In these pioneering studies, Albert et al. showed

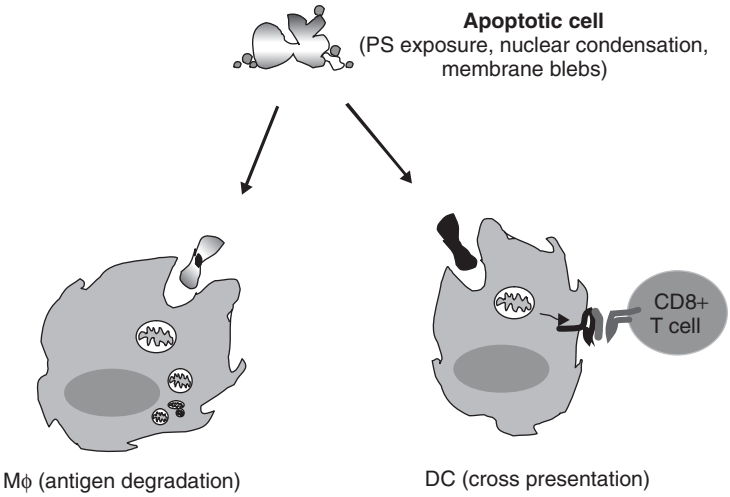


FIGURE 5. Macrophages and dendritic cells process apopotic cells differently. Upon their ingestion, DCs and macrophages process antigen differently. Whereas macrophages degrade internalized material in phagolysosomes, DCs can process antigens in a class I-restricted manner to cross-present or cross-tolerize antigen to T cells.

in vitro that immature peripheral DCs could stimulate antigen-specific CD8+ CTLs following engulfment of apoptotic influenza-infected thymocytes in the presence of exogenous maturation signal (106).

An important question that remains is under what conditions does it become appropriate to cross-present antigen from apoptotic cells? In the aforementioned arguments, maintenance of tissue homeostasis and prevention of autoimmunity would favor engulfment followed by noninflammatory events, in which either immune ignorance (degradation of apoptotic material), or immune tolerance (the recognition of self and inducing anergy or T-cell depletion) would ensue. Recent studies suggest that mature DCs expressing costimulatory molecules are required for induction of both immunity and tolerance, a choice mediated in part by the presence or absence of $T_H$ cells interacting with the mature DC (107). Therefore, engulfment of apoptotic cells by immature DCs, followed by maturation, can either cross-tolerize or cross-present antigens to CD8+ CTLs, depending on yet unknown signals. These signals will likely reflect the environment in which the apoptotic cells are engulfed, the inflammatory status of the tissue, and possibly unique epitopes/opsonizing factors on the surface of the apoptotic cell. Nevertheless, the implication that under certain conditions DCs can cross-present viral or tumor antigens to CTLs is potentially astounding if it can be directed toward the development of anticancer- or antiviral-based DC vaccines. Clearly, one of the long-term goals of apoptotic cell–phagocyte interactions will be toward modulating the immune response. In the long term, gene therapy and cellular manipulations of apoptotic cells and DCs might prove valuable for the development of anticancer therapies.

## Trafficking of Apoptotic Cells

A final important area of consideration with respect to engulfment centers on the trafficking of apoptotic material following internalization. Phagocytosis of apoptotic cells is initiated by binding to the phagocyte surface, leading to reorganization of the cortical actin cytoskeleton mediated in part by the activation of Rho- and Rab-family GTPases and products of the PI3-kinase pathway (108) in the phagocyte. The nascent phagosomes lack the ability to degrade apoptotic cells, but instead require a series of maturation steps in which the phagosome is remodeled and finally becomes a late endosome or lysosome that degrades engulfed material at acidic pH. One of the new and exciting findings with respect to phagosome function comes from both cell biology studies and a proteosome analysis that show the early phagosome is formed by direct fusion of the plasma membrane with endoplasmic reticulum (ER) membranes (109). ER-mediated phagocytosis not only provides a framework to explain the source of membrane required for

engulfing large particles, but may also offer an explanation for how antigens from apoptotic cells can be presented on class I MHC molecules as described in the preceding section (110). Finally, it also interesting to speculate that internalized apoptotic cells may traffic different routes, and in so doing, regulate different immunological outcomes. For example, it has been suggested that the trafficking of apoptotic cells in macrophages and dendritic cells is different, whereby macrophages degrade ingested antigen and dendritic cells cross-present or cross-tolerize antigen (73) (Fig. 5). It is also interesting that these cells express different integrins (αvβ3 on macrophages and αvβ5 on dendritic cells) that have subtly different regulatory properties. For example, αvβ3 integrin and αvβ5 integrin signal FAK and PKC differently (111), and result in differential trafficking of adenovirus (AdV) (112). In this capacity, αvβ5 integrin directs AdV to the cytoplasm, while αvβ3 integrin directs AdV to the lysosomal compartment. Clearly, an important future direction with respect to the phagocytosis of apoptotic cells will be to discern whether different receptors on the phagocyte surface direct differential trafficking or influence phagosome maturation.

## Summary

There is a great deal of experimental evidence now indicating that cell death can no longer be viewed as an endpoint. Specific ligands on apoptotic cells interact with receptors on phagocytes and trigger complex signaling events that lead to internalization and intracellular trafficking of the ingested material. One of the most exciting new ideas that has emerged over the past several years is that phagocytosis has immunological value. The uptake of apoptotic cells by macrophages and DCs affects several aspects of immune function, including inflammation and antigen presentation. There is also great hope that phagocytosis pathways can be manipulated to modulate the immunological response in disease situations. Harnessing the power of apoptotic cells within the context of human disease and gene therapy is an important but feasible challenge for the next several years.

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CHAPTER

14

# CELL CYCLE GENES: pRb AND p53

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## CELL DIVISION AND CELL DEATH: A SIMPLIFIED MODEL FOR LIFE AND DEATH

Cell division is the process by which two daughter cells are reproduced from a single cell. Two types of division may occur: mitosis and meiosis. Meiosis produces germ cells with a haploid set of chromosomes by one round of DNA replication and two rounds of cell division. Mitosis, however, takes place in somatic cells and generates two diploid daughter cells that are identical to the original cell. The orchestrated sequence of cellular processes by which one mother cell grows and divides into two daughter cells is termed cell division cycle, or cell cycle. In multicellular organisms, a precisely regulated balance between cell division and cell death is essential for normal development and homeostasis.

A typical cell cycle consists of four phases: a G1 phase, which is the first gap; an S phase, in which DNA synthesis takes place; a G2 phase or second gap; and an M phase, otherwise known as the mitotic phase. The G1-S-G2 phases are collectively termed interphase. Based on morphological changes, the mitotic phase can be further divided into prophase (DNA condensed to form sister chromatids), metaphase (sister chromatids migrate to the equatorial plane of the cell and are oriented in the center of the mitotic spindle), anaphase (simultaneous separation of all the sister chromatids at their centromeres), and telophase (chromosomes uncoil and new nuclear membranes

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form). In addition, G0 phase is used to refer to postmitotic, nondividing cells that exit the cell cycle and are at a resting state. G0 cells may reenter the cell cycle under certain conditions. The duration of the cell cycle varies in different cell types and this variation is generally caused by the G1 phase. Typically, a rapidly dividing human cell takes 24 hours to finish a complete division cycle.

A cell is a complex entity that consists of hundreds of thousands of macromolecules, including, but not limited to, lipid structure, proteins, and most importantly, the nucleic acids. To grow and divide, a cell needs to produce these macromolecules. Among these productive activities, the accurate duplication of DNA is the most important issue in cell division. To achieve this, a cell demands coordinated expression of cellular genes encoding mitotic growth factors and receptors, proteins involved in mitotic signaling pathways (most of them are proto-oncogenes), transcriptional factors, regulatory proteins that control cellular processes, and enzymes or other components for biological synthesis and metabolism, and finally, structure proteins. In this chapter, however, we will confine our discussion to genes proven to be essential for cell division, with a special focus on genes encoding cell cycle regulatory proteins and their functional regulation.

When a cell reproduces itself, a fundamental principle is the accurate transmission of genetic material from the mother cell to daughter cells. The accuracy of reproduction and transmission of genetic information depends on at least the following aspects: faithful DNA replication, effective DNA repair, proper spindle assembly, and successful chromosome segregation. To ensure accuracy, sequential events of the cell cycle are well coordinated and strictly controlled. On the one hand, downstream events will not occur until an upstream event has been completed successfully. On the other, an upstream event will not repeat itself unless the downstream events have taken place. Several checkpoints have evolved to control the cell cycle (Murray, 1992, 1994). The first identified checkpoint occurs at late G1, which is called "start" in yeast and "restriction point" in mammalian cells. Cells that have passed through start or restriction point initiate DNA replication and commit to division. Cells with damaged DNA in G1 cannot initiate DNA replication, and those with incompletely replicated DNA in G2 cannot enter mitosis (Hartwell and Weinert, 1989; Murray, 1992, 1994). Anaphase does not start until all the chromosomes have been adequately aligned on the spindle.

Over the past few decades, many efforts have been made to understand cell cycle control. It is generally agreed that the activity of E2Fs, a family of transcriptional factors, is essential for G1/S transition and cell cycle progression (Helin, 1998). The activity of E2Fs is governed by pRb-family tumor suppressors, whose function is controlled by phosphorylation (Sang et al., 1995). Phosphorylation of pRb-family proteins is a function of cyclin-dependent kinases (Cdks), which are activated by association with cyclins

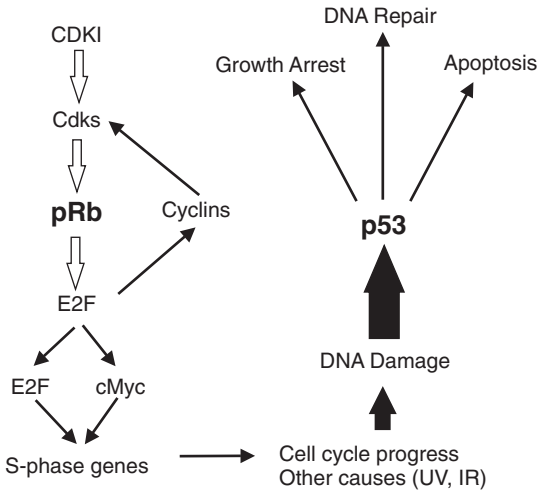


FIGURE 1. General roles of p53 and pRb in cell cycle regulation and apoptosis. Solid arrows: stimulating or up-regulating. Open arrows: inhibitive or repressive.

but inhibited by Cdk inhibitors (CDKI) (MacLachlan et al., 1995). The protein levels of cyclins oscillate during the cell cycle as a result of de novo synthesis at one stage, and of destruction by the ubiquitination system at another stage of the cell cycle (Koepp et al., 1999). Functional regulation of cell cycle proteins involves posttranslational modifications such as phosphorylation/dephosphorylation, acetylation/deacetylation, and multiple feedback loops. The tumor suppressor p53 serves as a "quality control" protein that continuously monitors the chromosome replication and possibly other cell division processes, and induces growth arrest or apoptosis in response to DNA damage or other genomic alteration to ensure genomic integrity and stability (Lane, 1992). A simplified, actually oversimplified, model is given in Fig. 1 and more details will be discussed later.

## CDC Genes: From *S. cerevisiae* to *H. Sapiens*

Yeast, especially budding yeast, *S. cerevisiae*, has contributed so much to our current understanding of the cell cycle that we cannot ignore them whenever we discuss this topic. Extremely well-designed studies in the genetic analysis of yeast cell cycles have identified several dozens of genes that are essential for cell division, demonstrated by the fact that mutation of each of these genes leads to cell cycle arrest at various stages of the cell cycle. These

genes have been termed cell division cycle genes. Earlier work about yeast cell cycle and cell division cycle genes have been summarized (Murray and Hunt, 1993). Because cell division cycle genes have been identified from both *S. cerevisiae* and *S. pombe*, and because homologues of most of these genes have been identified from mammals, the terms used for these genes and their products are somehow confusing. For clarity, we will follow the current consensus to indicate cell division cycle genes identified in *S. cerevisiae* as *CDC*, the corresponding mutants as *cdc*, and the proteins encoded by *CDC* genes as Cdc proteins. We will term cell cycle genes identified in fission yeast as *cdc+*, the mutants as *cdc–*, and the protein products as Cdcp.

Cloning and characterization of *CDC* and *cdc+* genes reveal that most *CDC* and *cdc+* gene-encoded proteins can be classified into several groups: (1) proteins involved in DNA synthesis and DNA repair; (2) enzymes essential for protein degradation; (3) protein kinases (including Cdks) and protein phosphatases that regulate protein functions by altering the phosphorylation status; (4) proteins involved in signal transduction; (5) proteins involved in energy metabolism, transcription, and protein translation; and (6) structure proteins required for cytokinesis and morphorgenesis. A complete list of CDC genes is provided in Table 1 for reference.

As DNA replication is a key process for cell division, it is not surprising to see that some *CDC* genes are involved in DNA synthesis and/or DNA repair. *CDC2* (*POL3*) encodes the catalytic subunit of DNA polymerase delta, and its importance is demonstrated by the fact that *cdc2ts* mutants arrest in late S phase when cultured at a restrictive temperature (Francesconi et al., 1993). Another *CDC* gene, *CDC17* (*POL1*), encodes DNA polymerase α, which is implicated in telomere homeostasis during DNA replication (Carlson and Hartwell, 1985; Adams Martin et al., 2000). Mutation of *CDC17* leads to cell arrest at mid to late S phase.

During a cell cycle, one, but only one, round of DNA replication is initiated. This is controlled by an important initiator encoded by *CDC6*, which is homologous to *cdc18* in fission yeast (Nishitani and Nurse, 1995) (Fig. 2). In collaboration with Cdt1, Cdc6/Cdc18p loads the minichromosome maintainence (MCM) proteins, which function as initiators of DNA replication (Tye, 1999), onto chromatin at the end of mitosis, thus licensing the DNA to replicate (Nishitani et al., 2000). As an essential component of the prereplication complex, the Cdc6/cdc18p level is tightly regulated during the cell cycle and peaks at G1-S transition. Cdc18p is controlled transcriptionally by Cdc10p in fission yeast, and the human homologue Cdc6 is controlled by the E2F family of transcriptional factors (Nishitani and Nurse, 1995; Hateboer et al., 1998). After G1-S transition, Cdc6/Cdc18p protein is phosphorylated by cyclins/Cdks that promote the degradation of Cdc6/Cdc18p (Baum et al., 1998; Jallepalli et al., 1997). Overexpression of Cdc6/Cdc18p results in a repeated round of DNA replication without mitosis (Nishitani and Nurse, 1995).

TABLE 1. SUMMARY OF CELL CYCLE GENES*

| *S. cerevisae* | *S. Pombe* | Mammal | Product features | General Function | Regulation | Cell Cycle Defects in Mutants |
|---|---|---|---|---|---|---|
| CDC2/POL3 | | | Catalytic subunits of DNA polymerase III | DNA replication | | Late S |
| CDC3 | | | Septin, budding ring formation | Cytokinesis | | Cytokinesis |
| CDC4 | | SCFcdc4 | Ubiquitin ligase | Protein degradation | | Defects in DNA replication & spindle pole body separation |
| CDC5/PKX2 | | | Kinase, phosphorylates Scc1 | Cytokinesis | | Arrest before cytokinesis |
| CDC6 | cdc18 | | Initiator of DNA replication, MCM-loading protein | DNA replication licensing | E2Fs (+), SCF degradation (–) | Late S phase arrest |
| CDC7 | | | Protein kinase, initiation of DNA replication | DNA replication initiation | Dbf4 (+) | G1 arrest |
| CDC8 | | | Thymidylate kinase | DNA replication | E2Fs (+) | Cease of DNA synthesis |
| CDC9 | cdc17 | | DNA ligase | DNA repair | Induced by DNA damage | G2 arrest with damaged DNA |
| CDC10 | | | Septin, budding ring formation | Cytokinesis | Cdc14 | Cytokinesis |
| CDC11 | | | Septin, budding ring formation | Cytokinesis | Cdc14 | Cytokinesis |
| CDC12 | | | Septin, budding ring formation | Cytokinesis | Cdc14 | Cytokinesis |
| CDC13 | | | Single-strand telomere-binding protein | DNA replication | | G2 arrest with damaged DNA |

TABLE 1. CONTINUED

| *S. cerevisae* | *S. Pombe* | Mammal | Product features | General Function | Regulation | Cell Cycle Defects in Mutants |
|---|---|---|---|---|---|---|
| CDC14 | | | Tyrosine phosphatase | Exit mitosis | Cdc15(+), | Mitosis with separated masses of DNA |
| CDC15 | cdc7 | | Protein kinase, phosphorylates Cdc14 | Exit mitosis | Cdc14(+) | Mitosis with a long spindle and separated masses of DNA |
| CDC16 | | APC6 | Subunit of APC | Protein (cyclin) degradation | Cdc20, CDH1(+), Emi1, MAD2, 2B (–) | Mitosis with a short spindle and unseparated chromsome |
| CDC17/POL1 | | | DNA pol alpha | DNA replication | | Arrest at mid to late S |
| CDC19/PYK1 | | | Pyruvate kinase, carbonhydrate metabolism | Metabolism | | Stop grow prior to Start |
| CDC20 | | | APC-activating protein | Protein degradation | Emil(–) | Mitosis with abnormal microtubules |
| CDC21/TMP1 | | | Thymidylate synthetase | DNA replication | | Stop DNA synthesis |
| CDC23 | | APC8 | Component of APC | Protein degradation | See CDC16 | |
| CDC24/CLS4 | Scd1 | | GDP/GTP exchange protein for Cdc42 | Signal transduction | Subcellular localization | Fail to bud |
| CDC25 | | | Guanine nucleotide exhange protein, regulates Cdc35 | Signal transduction | | Stop growth prior to Start |
| CDC26 | | | Heat-inducible subunit of APC | Protein degradation | See CDC16 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| CDC27 | | APC3 | Subunit of APC | Protein degradation | See CDC16 | |
| CDC28 | cdc2 | cdc2/cdk1 | Prototype of cyclin-dependent kinases | Promoting cell cycle | Cyclins, Sic1, Swe1 kinase | G1 arrest |
| CDC30 | | | Phosphoglucose isomerase | Metabolism | | Mitosis |
| CDC31 | | | Calcium-binding protein | Spindle pole body duplication | | Failure in spindle pole body duplication |
| CDC33 | | eIF-4E | Eukaryotic initiation factor | Translation | | G1 arrest |
| CDC34 | | | Ubiquitin-conjugating enzyme | Protein degradation | | DNA synthesis, spindle pole body separation |
| CDC35/CYR1 | | | Adenylate cyclase, increase cAMP | Signal transduction | Cdc25 | Arrest prior to Start |
| CDC36/NOT2 | | | Global transcriptional regulator | Transcription regulation | | Activate pheromone signaling and arrest at G1 |
| CDC37 | | | Chaperone, stabilizes Cdc28/Cln | Protein folding and maturation | | Similar to cdc28ts |
| CDC39/NOT1 | | | Global transcriptional regulator | Transcription regulation | | |
| CDC40/PRP17 | | | Pre-mRNA splicing factor? | DNA repair, RNA splicing | | Arrest with partially replicated DNA, effective in DNA repair |
| CDC42 | | | Small G protein | Signal transduction | Cdc24 (+), Cdc43 (–) | Fail to bud |
| CDC43 | | | Subunits of geranylgeranyltransferase that modifies Cdc42 | Protein modification | | Fail to bud |
| CDC44 | | RFC1 | DNA-dependent ATPase, accessory protein for DNApol. | DNA replication | | G2 arrest with large budded cells |

TABLE 1. CONTINUED

| *S. cerevisae* | *S. Pombe* | Mammal | Product features | General Function | Regulation | Cell Cycle Defects in Mutants |
|---|---|---|---|---|---|---|
| CDC45 | | | Promotes transition from prereplication complex to RC | DNA replication | | Fail to initiate DNA synthesis |
| CDC46 | | MCM5 | ORC-binding protein | DNA replication initiation | | Fail to initiate DNA synthesis |
| CDC47 | | MCM7 | ORC-binding protein | DNA replication initiation | | Fail to initiate DNA synthesis |
| CDC48 | | VCP | Oligomeric ATPase, ER fusion? | ? | | G2 arrest with a single-spindle pole body |
| CDC50/YCR094w | | | ? | Transcriptional regulation? | | Late G1 arrest |
| CDC53 | | Cul1 | Cullin 1, component of SCF ubiquitin ligase complex | Protein degradation | | |
| CDC54 | cdc21 | MCM4 | DNA helicase | DNA replication | | |
| CDC55 | | | β subunits of protein phosphatase 2A | Cytokinesis, kinetochore/spindle checkpoint | | Defects in cytokinesis |
| CDC60 | | | Leucyl-tRNA synthetase | Translation | | Arrest prior to start |
| CDC64 | | | Alanyl tRNA synthetase | Translation | | G1 arrest |
| CDC68/SPT16 | | | Transcription, elongation, chromosomal structure | Transcriptional regulation | | Arrest at late G1 |
| CDC72/NMT1 | | | N-myristol transferase that modifies Scg1 | Protein modification | | G1 arrest with constitutive pheromone signaling |

*References were cited in the text. Early information was based on Murray and Hunt, 1993.

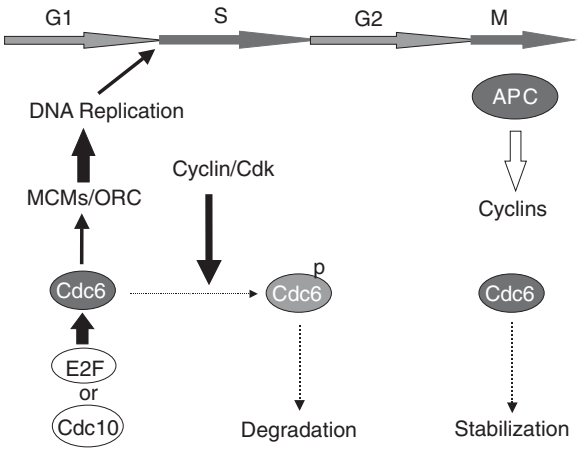


FIGURE 2. Cell cycle-dependent regulation of Cdc6 activity. Broken arrows indicate the change of status or fate.

Another group of genes involved in the initiation of DNA replication includes *CDC46*, *CDC47*, and *CDC54* (*cdc21* in fission yeast), which encode members of minichromosome maintenance proteins (MCM5, MCM7, and MCM4, respectively), the key components of the replication initiation complex that binds to the origin recognition complex (ORC) and initiates DNA synthesis in all eukaryotes (Tye, 1999) (Fig. 2). *CDC45* encodes a polypeptide that binds to MCMs and is essential for the initiation of DNA replication too (Hopwood and Dalton, 1996; Tercero et al., 2000). *CDC8* and *CDC21* encode thymidylate kinase and thymidylate synthetase, respectively, and are essential for DNA replication (Murray and Hunt, 1993). *CDC9* encodes a DNA ligase involved in DNA repair at G2 phase (Murray and Hunt, 1993). Another gene involved in DNA replication and DNA repair at G2 phase is *CDC13*, which encodes a protein that binds to the single-stranded telomere, thus protecting the telomere from degradation, and recruits telomerase (Nugent et al., 1996; Pennock et al., 2001). *cdc13* mutant arrests at G2 with damaged DNA (Murray and Hunt, 1993). *cdc40* mutant shows defect in DNA repair and arrest at S phase with partially replicated DNA (Murray and Hunt, 1993), and *CDC40*-encoded protein has been implicated in pre-mRNA splicing (Ben-Yehuda et al., 2000). *CDC44* encodes one of the five subunits of replication factor C (RFC). RFC interacts with the proliferating cell nuclear antigen (PCNA), which plays a role in both DNA replication and repair (Cullmann et al., 1995; McAlear et al., 1994). Cdc68/Spt16 forms a heterodimer with partners and acts in both DNA replication and

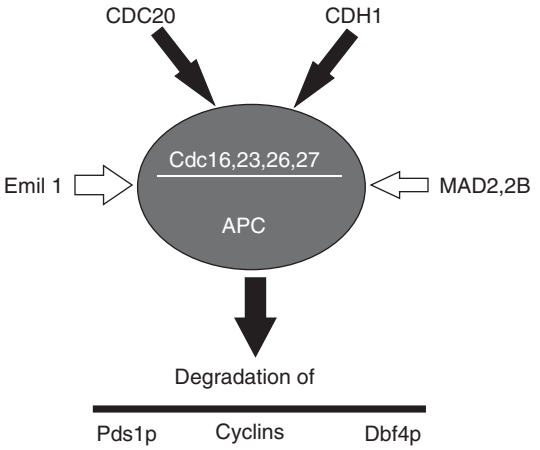


FIGURE 3. Role and regulation of APC (cyclosome) ubiquitinase activity in cell cycle. APC is a multi-unit complex composed of Cdc16, 23, 26, 27. Upon activation by Cdc20, it promotes the degradation of cell cycle regulators such as cyclins.

transcription (John et al., 2000; Formosa et al., 2001). In addition, some other genes, such as *CDC7*, encode regulatory protein that plays an indirect role in DNA replication.

As the cell cycle progresses, expression levels of many proteins such as cyclins, Cdc6, and so on are regulated dynamically. This regulation not only depends on de novo transcription and translation, but also requires protein degradation (Koepp et al., 1999). The first step for protein degradation is to mark the proteins to be degraded by ubiquitination systems (Hershko and Ciechanover, 1998). Two protein complexes have been shown to be essential for cell-cycle-regulated ubiquitination of proteins. The first one is termed anaphase-promoting complex (APC) or cyclosome (Page and Hieter, 1999), which consists of multiple subunits including protein products from *CDC16*, *CDC23*, *CDC26*, and *CDC27* (Fig. 3). Functioning as a cell-cycle-regulated ubiquitin-protein ligase, cyclosome is essential for mitosis in a wide range of eukaryotes (Lamb et al., 1994; King et al., 1995; Zachariae et al., 1996; Yu et al., 1998). In addition, *CDC20* encodes the activating subunit of APC (Vinsitin et al., 1997; Fang et al., 1998a, 1998b). Other APC regulators include the activator CDH1 and inhibitors Emil1, MAD2, and MAD2B (Reimann et al., 2001; Chen and Fang, 2001). APC targets the anaphase inhibitor Pds1p and cyclins for destruction, a function indispensable for cells to pass through anaphase and to exit mitosis (Shirayama et al., 1998; Noton and Diffley, 2000; Strohmaler et al., 2001). Another complex that is essential for cell cycle progression is SCF (Skip1-Cullin-F-box factor) (Deshaies, 1999) (Fig. 4). Cdc4 complexes with Cdc34 (ubiquitin-conjugating enzyme) and Cdc53, and this

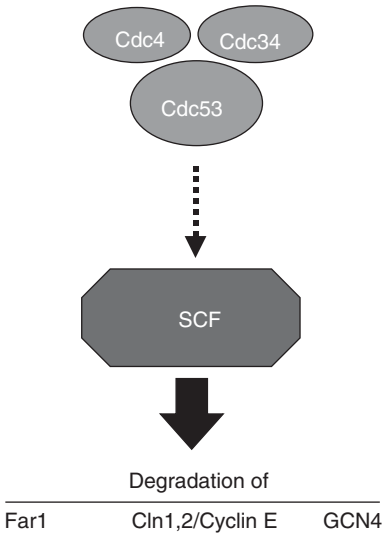


FIGURE 4. Role of SCF in cell cycle. Similar to APC, SCF is another multi-unit protein complex with ubiquitinase activity and controls the degradation of several key cell cycle regulators.

complex possesses ubiquitin-protein ligase activity (Kamura et al., 1999; Chi et al., 2001) required not only for the degradation of FAR1 and GCN4 (Blondel et al., 2000; Meimoun et al., 2000), but also for the degradation of Cln1 and Cln2, two G1 cyclins that regulate Cdc28 activity (Murray and Hunt, 1993) in yeast. *cdc4ts* mutant cannot initiate DNA synthesis and fails to separate the spindle pole body at restrictive temperatures (Murray and Hunt, 1993). The human homologue of *CDC4* encodes SCF(Cdc4), a member of the F-box family that interacts with hsCdc34 and targets cyclins (Strohmaler et al., 2001). Cdc53 is one of the core subunits of the SCF complex called cullin (Seol et al., 1999).

The third group of *CDC* genes code for various protein kinases and protein phosphatases involved in the regulation of protein functions by phosphorylation. The kinases and phosphatases form multiple protein kinase cascades and feedback regulation pathways. The most extensively studied one is Cdc28 kinase that associates with cyclins and will be discussed in more detail later. Cdc28 is the homologue of Cdc2p in fission yeast whose activity is essential for cell cycle progression at multiple stages in mammalian cells. *CDC5* encodes a pololike protein kinase whose function is indispensable for cytokinesis (Murray and Hunt, 1993). At the onset of anaphase in yeast cells, a caspase-related protease, separase, destroys the link between sister chromatids by cleaving the cohesin subunit Scc1. Cdc5 phosphorylates

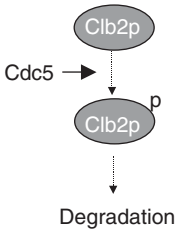


FIGURE 5. Role of Cdc5 in the degradation of Clb2p.

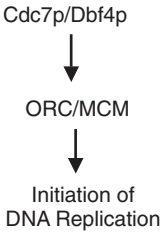


FIGURE 6. Cdc7p kinase activity stimulates the initiation of DNA replication.

serine residues adjacent to cleavage sites of Scc1 and enhances the cleavage (Alexandru et al., 2001). It is likely that this is a conserved mechanism which exists in higher organisms as well. Cdc5 is also involved in the proteolysis of Clb2p (Shirayama et al., 1998) (Fig. 5). Cdc7 was first identified as a serine/threonine protein kinase. The kinase activity is regulated by its association with Dbf4p, a cyclinlike regulatory subunit that is targeted for cell-cycle-regulated degradation by APC (Weinreich and Stillman, 1999; Ferreira et al., 2000). The Dbf4p/Cdc7 complex binds the origin recognition complex (ORC), and phosphorylates members of MCMs and other proteins essential for initiation of DNA replication, thus promoting DNA replication during the S phase (Pasero et al., 1999; Weinreich and Stillman, 1999; Jiang et al., 1999) (Fig. 6). Cdc15 protein kinase is a major component of the mitotic exit network (MEN), which consists of several other proteins such as Lte1, Tem1, Cdc5, Dbf2/Dbf20, and Mob1 (Mah et al., 2001). *cdc15* mutants arrest in

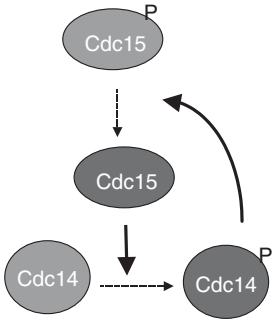


FIGURE 7. Positive feedback regulates Cdc14 and Cdc15 kinase activity. Phosphorylated Cdc15 is inactive. Upon dephosphorylation by Cdc14, Cdc15 is actived and phosphorylate Cdc14. In turn, phosphorylation of Cdc14 activates Cdc14 phsophotase, activity which dephosphory lates Cdc15.

mitosis with a long spindle and two separated masses of DNA. Cdc15 serves as both an activator and substrate for Cdc14, a tyrosine phosphatase required for mitosis (Wan et al., 1992; Visintin et al., 1998, 1999; Shou et al., 1999) (Fig. 7). On one hand, Cdc15 functions in the mitotic exit network to activate Cdc14 phosphatase, and Cdc14-mediated dephosphorylation triggers the inactivation of mitotic cyclins. On the other, Cdc14 also activates Cdc15 activity by dephosphorylation, thus forming a positive feedback (Mah et al., 2001; Jaspersen and Morgan, 2000; Xu et al., 2000). Cdc55 is the counterpart of the beta subunit of protein phosphatase 2A with multiple functions in mitosis and is required for the kinetochore/spindle checkpoint, whereas mutants show defects in cytokinesis (Healy et al., 1991; Wang and Burke, 1997; Yang et al., 2000). Finally, *CDC37* is genetically related to *CDC28* and is required for Cdc28 function. *cdc37* mutants show a phenotype similar to Cdc28ts (Murray and Hunt, 1993). The polypeptide encoded by *CDC37* is a molecular chaperone and specifically targets Cdc28 and several other protein kinases, stabilizes Cdc28, and facilitates the formation of Cdc28/cyclin complex (Stepanova et al., 1996; Kimura et al., 1997; Farrel and Morgan, 2000) (Fig. 8).

Signal transduction is required for cell cycle regulation at all phases during the cell cycle. A traditional example is that mating factor pheromone signaling blocks cell division (MacLachlan et al., 1995). The *CDC* genes also are involved in transducing signals to regulate the cell cycle. *CDC72* (*NMT1*) encodes N-myristol transferase, which blocks mating factor signaling by modifying Scg1, the alpha subunit of the trimeric G protein that is coupled to the mating factor receptor. *cdc72* mutants show constitutive activation of the mating factor signaling pathway and cell cycle arrest (Murray and Hunt, 1993). Both *CDC25* and *CDC35* have been implicated in the cAMP-protein kinase A signaling pathway (Fig. 9). *CDC35* (*CYR1*) encodes an adenylate

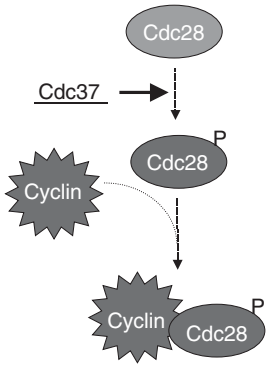


FIGURE 8. Phosphorylation of Cdc28 by Cdc37 promotes the formation of active cyclin/Cdc28 complex.

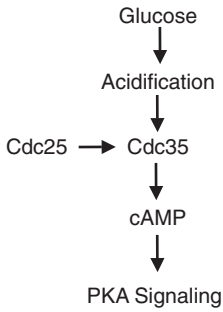


FIGURE 9. Role of Cdc25 and Cdc35 in the PKA signaling pathway.

cyclase and affects intracellular cAMP levels (Casperson et al., 1985). In response to glucose and acidification of the environment, Cdc35 increases the cAMP levels in cells and promotes glucose uptake by cells (Oehlen et al., 1994). Cdc25 is a guanine nucleotide exchange protein that regulates Cdc35 activity (Engelberg et al., 1990). Either *cdc35* or *cdc25* mutation causes growth arrest prior to the "start" of the cell cycle and a failure in increase of cell mass. Rho-type GTPases control many cytoskeletal rearrangements. Cdc42 is a small GTPase that is involved in polarity establishment during bud for-

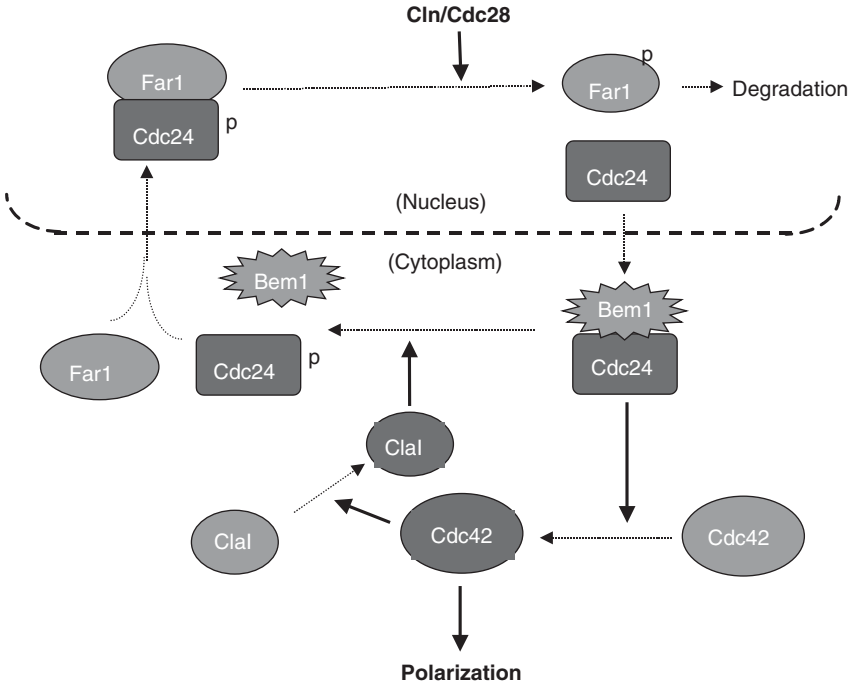


FIGURE 10. Control of polarization by cell cycle regulators.

mation (Fig. 10). Cdc24 is the GDP/GTP exchange factor for Cdc42 and is sequestered in the nucleus by Far1. The activation of Cdc28/Cln upon bud emergence triggers the degradation of Far1 and the relocation of Cdc24 from the nucleus to the polarization site, where it binds directly to Bem1 and activates Cdc42 (Shimada et al., 2000). Activated Cdc42 and its effectors polarize the cytoskeleton (Gulli et al., 2000). One of the Cdc42 effectors, Cla1, phosphorylates Cdc24 and releases it from Bem1, thus forming a negative feedback control loop (Bose et al., 2001). Human Cdc24 also catalyzes guanine nucleotide exchange on human Cdc42 (Bender and Pringle, 1991; Butty et al., 2002). Cdc43 is a subunit of geranylgeranyltransferase that modifies Cdc42 function (Finegold et al., 1991).

The fifth group of *CDC* genes consists of genes involved in basic energy metabolism, transcriptional regulation, and protein translation. Defects in this group of genes usually lead to growth arrest prior to the start of the cell cycle and cells cannot increase in size. Two *CDC* genes are involved in energy

metabolism: *CDC19* (*PYK1*), which encodes pyruvate kinase (Moore et al., 1990), and *CDC30,* which encodes phosphoglucose isomerase (Dickinson, 1991). Cdc33 is the eukaryotic initiation factor 4E (eIF-4E) that binds to the cap at the 5′ end of mRNA. *CDC60* is a leucyl-tRNA sythetase (Hohmann and Thevelein, 1992). *CDC63* (*PRT1*) is essential for the initiation of protein synthesis (Hanic-Joyce et al., 1987). *CDC64* encodes Ala1p, an alanyl-tRNA synthetase (Wrobel et al., 1999).

Finally, components of the cell skeleton and organelles are equally essential for cell division, especially in the late cytokinesis phase. *CDC3*, *CDC10*, *CDC11*, and *CDC12* form a group of related genes whose products are required for assembling a ring at the neck to divide mother and daughter cells. Mutation of any of these genes leads to a failure in cytokinesis (Kim et al., 1991). *CDC31*-encoded small calcium-binding protein is required for spindle pole body duplication (Spang et al., 1993) and is homologous to human centrins (Middendorp et al., 1997). *CDC48* is homologous to oligomeric ATPase involved in membrane fusion and organelle biogenesis. *cdc48* mutants arrest in G2 with a single-spindle pole body and abnormally distributed microtubules (Murray and Hunt, 1993).

Cell division cycle genes also have been identified from fission yeast *S. pombe* and termed *cdc+* genes to distinguish them from *CDC* genes of *S. cerevisiae*. Some of the *cdc+* genes that have corresponding *CDC* homologues have been discussed, and other important genes are summarized in Table 2. Among these genes we now focus on *cdc10+* and *cdc25+.* cdc10p binds to Sct1 to form a heterodimer that functions as a transcriptional factor to promote the expression of S phase genes. Swi6 in *S. cerevisiae* is a homologue of cdc10p, whereas in mammalian cells E2F is the functional counterpart of

TABLE 2. Summary of Cyclin-dependant Kinases

| Cdks | Alias | Cell Cycle | Cyclin | Substrates |
|---|---|---|---|---|
| Cdk1 | Cdc2p/Cdc28 | G2/M | A, B | Histone H1, lamins |
| Cdk2 | | G1/S | A, E, J | DNA replication protein? |
| Cdk3 | | G1 | E2 | E2F? |
| Cdk4 | | G1 | D1, D2, D3 | pRbs |
| Cdk5 | | G0 | p35 | Tau, neurofilamin, NUDF |
| Cdk6 | | G1 | D1, D2, D3 | pRbs |
| Cdk7 | CAK/MO15 | ? | H | Cdks and CTD of PoIII |
| Cdk8 | | G1? | C | Cyclin H in TFIIH |
| Cdk9 | PITALRE/TAK/P-TEFb | Ubiquitous | K, T1, T2 | CTD |
| Cdk10 | PISSALRE | Ubiquitous | ? | Ets? |

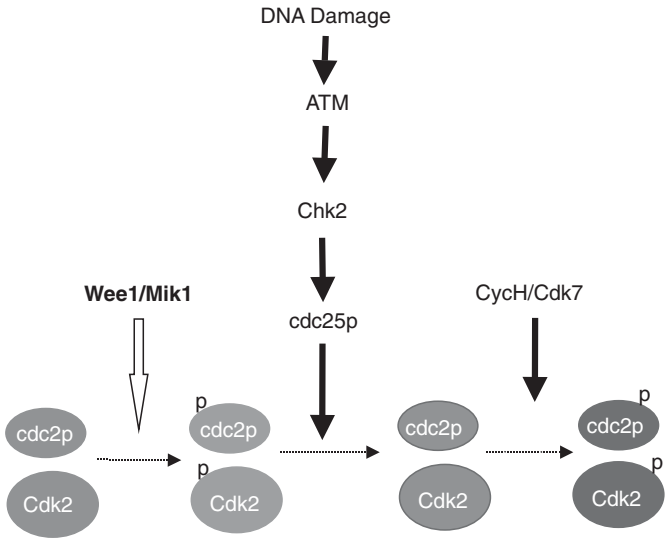


FIGURE 11. Regulating cdk activity by DNA damage signaling.

cdc10p. *cdc25+* encodes a tyrosine phosphatase, cdc25p, which dephosphorylates cdc2p and activates its kinase activity at late G1. In mammalian cells, three cdc25p homologues (Cdc25A, B, and C) have been identified. Cdc25A dephosphorylates and activates Cdk2, a process required for DNA synthesis. But in response to DNA damage, Cdc25A is phosphorylated by ATM-chk2 and degraded by the ubiquitin system. Loss of Cdc25A leads to a transient blockage of DNA replication, thus facilitating DNA repair (Falck et al., 2001) (Fig. 11).

## Cyclin and Cyclin-Dependent Kinases: the Promoting Engine of the Cell Cycle Machinery

Historically, three lines of investigation converged and eventually led to the identification of cyclins and cyclin-dependent kinases as the engine of cell cycle machinery. In the 1970s, a factor produced in mature eggs from frogs (*Xenopus laevis*) was found to be able to promote the maturation and division of immature oocytes and was termed maturation-promoting factor (MPF). Through the use of this oocyte maturation assay, MPF activity was detected in extracts from all cell types tested. In addition, it was observed

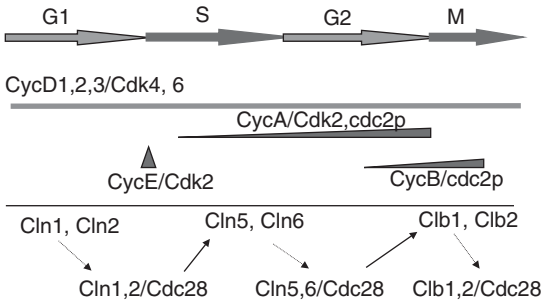


FIGURE 12. Fluctuation of cyclin/cdk activities during cell cycle.

that MPF fluctuated during the cell cycle with a peak activity as cells entered mitosis that dropped rapidly after the cell division was completed. In the 1980s, three lines of investigation resolved the biochemical nature of MPF. First, during the embryonic cell cycle of sea urchins, proteins characterized by cyclic accumulation and disappearance were observed and termed cyclins. Second, characterization of yeast mutations that led to cell cycle arrest isolated p34cdc2 (cdc2p) in fission yeast (Broek et al., 1991). Finally, the purification and identification of MPF revealed that MPF is a protein kinase complex which consists of cyclin B and cdc2p kinase. Although cdc2p is the catalytic subunit, its kinase activity depends on the regulatory subunit cyclin B. Now cdc2p is considered the prototype of cyclin-dependent kinases.

In budding yeast or fission yeast, a single Cdk encoded by *CDC28* or *cdc2*, respectively, is responsible for promoting the cell cycle by interacting with several cyclins at various phases (Nasmyth, 1993; Sherr, 1994) (Fig. 12). The association with phase-specific cyclins activates the kinase activity to promote the cells forward to the next phase. At the G1 phase, Cln3, a constitutive cyclin, promotes the accumulation of Cln1 and Cln2 and their association with Cdc28/cdc2p. Cln1 and Cln2 work cooperatively to promote the transition through the checkpoint called "start." After entering S phase, Cln5 and Cln6 replace Cln1 and Cln2 to associate with Cdc28 and promote progress through the S phase. At M phase, the mitotic cyclins Clb1 and Clb2 form complexes with Cdc28 kinase.

In mammalian cells, at least nine additional members of the cyclin-dependent kinase family have been identified and named, from Cdk2 to the newest Cdk9 and Cdk10 (Graña et al., 1994a, b; De Falco and Giordano, 1998; Kasten and Giordano, 2001) (Table 2). These Cdks associate with members

TABLE 3. Summary of Cyclins

| Cyclins | Cell Cycle | Associated Cdks | Regulation |
|---|---|---|---|
| A | S to M | Cdk2, Cdc2 | E2F (+), APC (–) |
| B1 | G2/M | Cdc2 | Transcriptional (+), Degradation (–) |
| B2 | G2/M | ? | Transcriptional (+), Degradation (–) |
| C | G1? | Cdk8 | ? |
| D1 | G1 | Cdk4, Cdk6 | Transcriptional (+), Degradation (–) |
| D2 | G1 | Cdk4, Cdk6 | Transcriptional (+), Degradation (–) |
| D3 | G1 | Cdk4, Cdk6 | Transcriptional (+), Degradation (–) |
| E | G1/S | Cdk2 | Transcriptional (+), Degradation (–) |
| F | G2/M | Cdk2? | |
| G | Ubiquitous? | ? | |
| H | Ubiquitous | Cdk7 | |
| I | Ubiquitous | ? | |
| J | | Cdk2 | |
| K | | Cdk9 | |
| T | | Cdk9 | |

of the cyclin family, including cyclin A, B1, B2, C, D1, D2, D3, E, F, G, H, I, J, K, and T, in a combinatorial manner to form diverse Cdk/cyclin complexes. These cyclin/Cdk complexes coordinately regulate the cell cycle and other cellular processes (Table 3). Although the essential roles of cyclin A, B, D, E, and H complexes in cell cycle regulation have been proven, it should be pointed out that a few members of the cyclin and Cdk families may not be directly involved in cell cycle control. These members obtained their names by their structural homology to, and/or by their ability to genetically complement, yeast deficiency in the cyclin genes or the *CDC28*/*cdc2* genes. The existence of multiple types of cyclin-dependent kinases and the intricate combination between cyclins and Cdks in multicellular organisms such as mammals reflect the complex regulation of cell division and perhaps of other cellular processes such as differentiation and development.

There are three members of D-type cyclins: D1, D2, and D3. Although they are cyclins, their protein levels generally do not oscillate through the cell cycle (Fig. 12). The association between D-type cyclins and Cdk4 or Cdk6 is responsible for G1 progression. Cyclin E protein begins to rise in mid G1 and peaks near the G1/S boundary. During the G1/S transition, cyclin E activates Cdk2 activity (Koff et al., 1992). Cyclin A is located in the nucleus of cells and appears late in G1 just before the start of DNA synthesis; it slowly increases in amount and associates with both Cdk2 and Cdc2p until the cells reach prophase. There are two forms of B-type cyclins: B1 and B2. Cyclin B1 binds only to cdc2p and appears in the cytoplasm late in S phase and is then

imported into the nucleus. Association of cdc2p kinase with cyclin B or cyclin A in G2 phase initiates mitosis (King et al., 1994).

Cyclin degradation is required for inactivation of cyclin/Cdk activity and for exit from mitosis. Nondegradable cyclins arrest cells in mitosis. Ubiquitination-dependent proteasome is involved in cyclin degradation. Both cyclin A and cyclin B have destruction boxes located at the N-terminus that are responsible for their rapid turnover at metaphase via a ubiquitin-dependent pathway such as the APC and SCF complexes.

In addition to its association with cyclins, phosphorylation regulates the kinase activity of Cdks. Wee-1 is a tyrosine kinase controlling the G2/M transition in yeast by phosphorylating tyrosine 15 of Cdc28/cdc2p, and a Wee1-like kinase existing in human cells phosphorylates Cdc2p at threonine 14 and tyrosine 15. This phosphorylation, however, is inhibitory (Fig. 11). *mik1* (mitotic inhibitory kinase gene) is closely related to *wee1* and encodes a kinase with a similar activity to Wee1 in fission yeast. Another protein kinase, CAK/MO15, also called Cdk7 (cyclin-H-dependent), directly phosphorylates Cdc2p and activates its kinase activity. Cdc25p, a protein phosphatase that specifically dephosphorylates Cdc2p at threonine 14 and tyrosine 15, controls the G2-M transition and plays a role as a mitotic inducer. Three Cdc2p-activating Cdc25p phosphatases have been identified in humans, namely, Cdc25A, B, and C, with a partially redundant function (Chen et al., 2001).

Cyclin/Cdks modify protein function by phosphorylation. Most of the substrates of cyclin/Cdks are cell-cycle-related proteins. First of all, one type of cyclin/Cdk complex may phosphorylate and regulate the kinase activity of another cyclin/Cdk complex, as in the case of cyclin H/Cdk7 (King et al., 1994; Tsai et al., 1994) (Fig. 11). The second group of substrates for cyclin/Cdk complexes are the pRb-family proteins, which are transcription regulators and play important roles in cell cycle control (Sang et al., 1995; Paggi et al., 1996) (Fig. 13). The third substrate group includes transcription factors and coactivators. DP1, the dimerization partner of E2F, for example, undergoes phosphorylation during cell cycle progression, possibly by cyclin A/Cdk2 (Krek et al., 1994). Phosphorylation decreases the DNA-binding activity of the DP1/E2F heterodimer (Bandara et al., 1994). Phosphorylation of the C-terminus of RNA polymerase II by Cdc2p controls the transition from transcription initiation to elongation. The fourth group of substrates consists of chromosome components such as histone H1 (Giordano et al., 1989, 1991). Chromatin consists of histone proteins. Two copies each of histone H2A, H2B, H3, and H4 form an octomer core around which DNA is wound. Histone H1 interacts with the outer surface of the DNA and promotes DNA packaging. Cyclin B/cdc2p complex phosphorylates histone H1, and this phosphorylation leads to the dissociation of H1 from chromatin. In addition, nuclear lamins are part of the nuclear envelope. Cyclin B/Cdc2p phosphorylates lamin B2 at two sites, triggering a cascade of reactions and

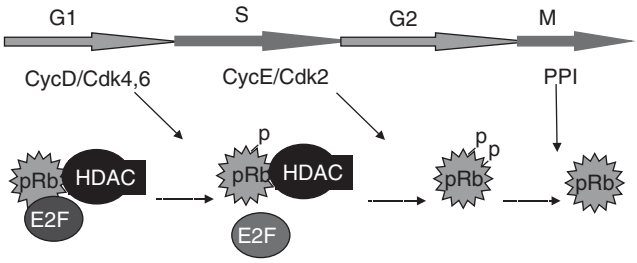


FIGURE 13. Functional fluctuation of pRb activity during cell cycle.

ultimately leading to the phosphorylation of lamin B2 at a third site, which promotes the nuclear envelope to break down. The mitotic spindle has three Cdc2p kinase sites and is phosphorylated only during mitosis. For some proteins, phosphorylation may serve as a signal for ubiquitination and ultimate degradation.

## CYCLIN-DEPENDENT KINASE INHIBITORS: BRAKES OF THE CELL CYCLE MACHINE

Several negative regulatory subunits of Cdks, for example, cyclin-dependent kinase inhibitors (CDKI), have been identified and can be categorized into two groups: the universal CDKI family that includes p21, p27kip, and p57kip, and the INK4 family, consisting of p16INK4a, p15INK4b, p18INK4c, and p19INK4d, that specifically targets Cdk4 and Cdk6.

The prototype of the first family of CDKI is p21 (Elledge and Harper, 1994; Hunter and Pines, 1994; MacLachlan et al., 1995). p21 was identified by several independent groups with different approaches. First, it was identified as a Cdk-interacting protein, Cip1 (Harper et al., 1993; Xiong et al., 1993a). A second group identified it as a 20KDa Cdk-associated protein, CAP20 (Gu et al., 1993). As a direct transcriptional target of p53, it was identified as wild-type p53-activated factor-1, WAF1 (El-Diery et al., 1993). Identification by various approaches reflects various aspects of p21 activity and function. As cell cycle regulators, kinases directly inhibited by p21 include cyclin D/Cdk2 and cyclin D/Cdk4 at G1, cyclin E/Cdk2 at the G1/S transition, and cyclin A/cdc2 and cyclin B/cdc2 during mitosis (MacLachlan et al., 1995). In addition, p21 has been found to inhibit DNA replication by its interaction with proliferating cell nuclear antigen (PCNA), which plays an essential role in both DNA replication and DNA repair (Waga et al., 1994).

Overexpression of p21 in cells inhibits cell growth that substantiates its role in cell cycle control (El-Diery et al., 1993). Mice deficient in p21 display neither developmental defects nor increased rate of spontaneous tumorigenesis; however, p21-deficient embryonic fibroblasts are partially refractory to G1 arrest induced by DNA damage (Brugaroslas et al., 1995; Deng et al., 1995).

Another universal Cdk inhibitor, p27kip1, was first identified in complexes with cyclin E/Cdk2 in transforming growth-factor-beta-induced, growth-arrested cells (Koff et al., 1993; Polyack et al., 1994). It is able to bind a broad range of Cdks and controls the G1/S transition (Firpo et al., 1994; Polyack et al., 1994). p57kip was first identified as a member of the p21 family and as a tumor suppressor (Lee et al., 1995; Matsuoka et al., 1995). Like p21, p57kip inhibits cell proliferation and promotes cell differentiation, and thus is responsible for cell cycle withdrawal and terminal differentiation. Mice deficient in p57 die perinatally within 1 day, and display short limbs and an inflated gastrointestinal tract.

The INK4 family of cyclin-dependent kinase inhibitors includes four members: p16INK4a, p15INK4b, p18INK4c, and p19INK4d, which are expressed in a tissue-specific manner. p16INK4a and p15INK4b are structurally and functionally related. p16INK4a, the prototype of this subgroup, was first identified as a 16 kDa protein associated with Cdk4 in transformed cell lines and as a melanoma tumor suppressor, MTS1 (Xiong et al., 1993b; Kamb et al., 1994). Both p15INK4b and p16INK4a are able to inhibit cyclin D/Cdk4 and cyclin D/Cdk6 (Hannon and Beach, 1994) through direct interaction and to act as G1 inhibitors. In human keratinocytes, p15 is significantly induced by treatment with TGF-beta transcriptionally (Hannon and Beach, 1994). In addition to p16, the same locus encodes another transcript from an alternative reading frame, p19ARF (Quelle et al., 1995), which is not a CDKI, but also has growth-suppressing activity, which is caused by sequesting MDM2, a protein that binds p53 and promotes the degradation of p53 to the nucleoli, thus stabilizing p53 (Fig. 14). p16-deficient mice are viable without major defects in development, but often develop spontaneous tumors, predominantly lymphoma and fibrosarcoma (Serrano et al., 1996). In addition, the mice are more sensitive to DMBA or UV-induced carcinogenesis. *p16*$^{-/-}$ MEFs have a much shorter doubling time. p18INK4c specifically targets Cdk6 at the G1 phase (Guan et al., 1994). Overexpression of p18INK4c induces growth suppression in *RB*+/+ cells but not in *RB*−/− cells.

## The pRb Family Tumor Suppressors: Targets of Cyclin and Cyclin-Dependent Kinases

*RB1* is the prototype of tumor suppressor genes and was initially identified as a locus mutated in retinoblastoma, a childhood tumor of the retina (Lee

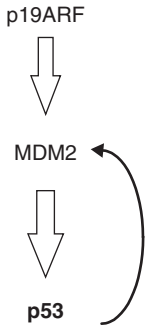


FIGURE 14. Basic regulatory loop of p53 activity.

et al., 1987). Mutations in both alleles of *RB1* not only exist in all retinoblastomas, but also in other tumors, such as osteosarcomas, small cell lung cancers, soft tissue sarcomas, breast cancers, bladder carcinomas, and prostate carcinomas. *RB1* transcript and the protein product pRb, a 105 kD nuclear phosphoprotein (Lee et al., 1987), are present in all normal tissues examined. In *RB1*−/− cell lines, microinjection of the pRb protein in early to mid G1, or transfection of the cDNA-encoding pRb, results in a reversal of the tumor phenotype and arrests cells at G1 phase (Huang et al., 1988; Bookstein et al., 1990). These early findings show that pRb is a universal growth suppressor that functions at the G1 phase.

The function of pRb is regulated largely through phosphorylation by cyclin/Cdk complexes (Fig. 13). The phosphorylation status of pRb oscillates regularly throughout the cell cycle (Chen et al., 1989). At G0 and early G, pRb is hypophosphorylated, associates with histone deacetylase and E2F, and functions as a negative regulator of the cell cycle. As the cell cycle progresses, pRb is phosphorylated by cyclin/Cdk complexes at different steps: mid G1, S, and near the G2/M transition (De Caprio et al., 1992). At least cyclin D/Cdk4, cyclin D/Cdk6, and cyclin E/Cdk2 are responsible for the phosphorylation of pRb in vivo. Phosphorylation of pRb by cyclin/Cdk complexes at the G1 phase disrupts its association with histone deacetylase (HDAC) and E2F, thus inactivating pRb and removing pRb-mediated cell cycle block. At the late mitotic phase and just before reentry into G1 phase, pRb is converted to the hypophosphorylated state by Type I phosphatases (PP1) and becomes active. In addition, apoptotic proteases have been shown to be involved in the degradation of pRb (Kasten and Giordano, 1998).

Structurally and functionally related to pRb, p107 and p130/Rb2 share large regions of homology especially in the "pocket region" (Ewen et al.,

1991; Hannon et al., 1993; Mayol et al., 1993; Li et al., 1993), and all three members form the pocket protein family. The growth suppressive activity of the pocket protein family depends on the pocket regions to interact with E2F (Hiebert et al., 1992; Qin et al., 1992). Like pRb, the other two members undergo cell-cycle-regulated phosphorylation (Baldi et al., 1995; Mayol et al., 1993), associate with HDAC and E2F, and have growth suppressive properties (Ewen et al., 1991; Claudio et al., 1994; Stiegler et al., 1998).

pRb represses cell cycle progression by modulating the activity of the E2F family of transcription factors. At G0 and G1, unphosphorylated or hypophosphorylated pRb binds to E2F through the pocket region. This interaction blocks the expression of E2F target genes whose products are required for DNA synthesis and cell cycle progression (Zhu et al., 1993; Sala et al., 1994). p107 and p130/Rb2 also bind to E2F and function in a similar way (Cobrinik et al., 1993; Schwarz et al., 1993). However, each pocket protein has a different temporal profile of expression and interacts with different members of the E2F family during the cell cycle (Classon and Dyson, 2001). The binding of p130/Rb2 to E2F4,5 is detected predominantly during G0, and the binding of p107 is detected during G1 and S phase, while the binding of pRb is detected during late G1 and continues through S phase. In addition, recruitment of HDAC may enhance the repression of E2F activity (Ferreira et al., 1998; Stiegler et al., 1998). Interestingly, the role of pRb2/p130 in promoting apoptosis was suggested by recent reports (Bellan et al., 2002; Pucci et al., 2002). Viral oncoproteins, including the adenoviral E1A protein, the large T antigens of polyomavirus such as SV40, the human JC and BK viruses, and the E7 oncoprotein of HPV, specifically bind to and inactivate unphosphorylated or hypophosphorylated pRb, thus stimulating E2F activity. Therefore, the association between the viral proteins and pRb mimics the phosphorylation of pRb by cyclin-Cdk complexes and the genetic alteration of *RB1* in tumor cells.

E2F was originally identified as a DNA-binding factor that is required for adenoviral E1A-mediated induction of the viral E2 promoter. Five members of E2F family, E2F1 (Helin et al., 1992; Kaelin et al., 1992; Shan et al., 1992), E2F2 (Ivey-Hoyle et al., 1993; Lees et al., 1993), E2F3 (Lees et al., 1993), E2F4 (Beijersbergen et al., 1994; Ginberg et al., 1994; Sardet et al., 1995), and E2F5 (Hijmans et al., 1995; Sardet et al., 1995), have been identified and functionally characterized. Although E2F1–3 are transcriptionally active and can be repressed by Rb family, E2F4 and E2F5 are considered repressive and their repressive activity depends on p130/RB2 and p107. The sixth member of E2F family, E2F6, has been cloned but its function remains unclear. E2F family members form heterodimers with either of two dimerization partners, DP1 or DP2. Studies have revealed that DP1 is slightly phosphorylated by cyclin/Cdk complexes during early cell cycle progression and that its levels of phosphorylation increase during progression through the cell cycle. This increase in phosphorylation levels is associated with a decrease in

DNA-binding activity of the E2F/DP-1 heterodimer. A variety of genes that code for proteins involved in DNA replication are activated at the boundary between G1 and S of the cell cycle. Many of these genes contain E2F-binding sites at their promoter regions. Important E2F target genes include, but are not limited to, c-Myc/N-myc, dihydrofolate reductase (DHFR), thymidine kinase, DNA polymerase alpha, thymidylate synthase, PCNA, ORC1, cyclins and Cdks, pRb family, and E2Fs themselves. While E2F activity is required for S phase progression, it is proposed that uncontrolled, overactive E2F function leads to apoptosis (Fig. 1).

## p53: The Governor of Life and Death

p53 was identified as a cellular protein associated with SV40 large T antigen in SV40-transformed cells and was initially termed SV40-associated tumor antigen. Overexpression of wild-type p53 inhibits the growth of both normal and transformed cells, thus showing that it actually is a tumor suppressor (Finlay et al., 1989). Indeed, p53 mutations are present in more than 50% of human tumors. p53 function, however, is not limited to growth suppression. The current notion is that p53 is a multifunctional protein with a key role in the maintenance of genomic integrity so that not only can the cells and the organisms as a whole survive, but also live much better. To achieve that goal, p53 responds to various signals, integrates these signals, and coordinates cell growth, DNA repair, and apoptosis.

p53 has the structural characteristics of a transcriptional factor and functions in the form of a homotetramer. It interacts with the TATA-box binding protein (TBP) and p300/CBP through its transactivation domain (Seto et al., 1992; Mack et al., 1993; Avantaggiati et al., 1997; Sang et al., 1997; Giordano and Avantaggiati, 1999). As a sequence-specific DNA-binding protein, p53 can regulate gene transcription either positively or negatively. First of all, the universal Cdk inhibitor p21 (WAF1) is a transcriptional target of p53 (Fig. 15). Since p21 inhibits cyclin/Cdk complexes that are responsible for the inactivation of the pRb family through multiple-site phosphorylation, the coordinated control of the cell cycle by p53 and pRb has been proposed (Sang et al., 1995). In addition, the complex formation between p21 and PCNA suggests that p53 is involved in DNA repair, a notion substantiated by the identification of another p53 target gene, *gadd45*. *gadd45* belongs to a family of genes called "growth arrest to DNA damage" (*gadd*) genes whose expression is enhanced upon growth arrest induced by DNA damage (Fornace et al., 1989). Gadd45 recognizes altered chromatin state and modulates DNA accessibility to cellular proteins (Carrier et al., 1999). Gadd45 also stimulates the DNA excision repair pathway and plays a role in inhibition of the G1/S transition by interacting with PCNA. p53 induces apoptosis by transcriptionally controlling the expression of the *bax* gene that encodes a key promoter of

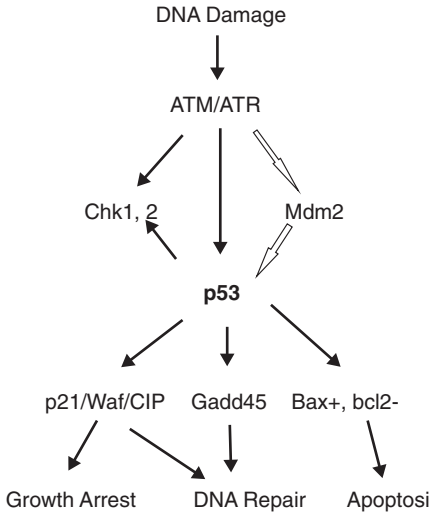


FIGURE 15. p53-response to DNA damage. Solid arrows: stimulatory. Open arrows: inhibitory.

apoptosis, and of the protooncogene bcl-2. Bax and Bcl-2 form either homodimers with themselves or heterodimers with each other to switch between apoptosis and survival (Oltvai et al., 1993). Increased p53 activity transcriptionally enhances the expression of *bax* but represses the expression of *bcl2*. In addition, the level of Mdm2 is transcriptionally enhanced by p53, providing negative feedback to regulate p53 (Momand et al., 1992; Barak et al., 1993; Oliner et al., 1993) (Figs. 14 and 15).

As a guardian of the genome, p53 continuously monitors the genomic integrity and responds to it accordingly. Exposure of cells to different kinds of DNA-damaging agents, such as UV, X-rays, ionizing radiation, and genotoxic chemicals, leads to p53 accumulation and activation. DNA damage also occurs during normal cell processes, including DNA replication, rearrangements, homologous recombination and chromosomal reshuffling during cell division, differentiation, spermatogenesis, and oogenesis. When DNA damages are detected, p53 transcriptionally regulates the expression of its target genes and arrests the cell cycle at G1 to repair the damaged DNA before replication can occur. At G2, genomic alteration caused by improper DNA replication could induce p53 and cause G2 arrest. 14-3-3ε, a protein factor that is involved in G2 arrest, is induced by DNA damage agents in a p53-dependent manner (Hermeking et al., 1997). In response to DNA damage, 14-3-3ε-deficient cells fail to arrest at G2, but die upon entering

mitosis (Chan et al., 1999). p53-induced cell cycle arrest provides the cells with enough time to repair the damaged DNA. Alternatively, if the DNA damage is too severe to be repaired, p53 triggers the apoptosis pathway to actively destroy mutant cells. By these means, it prevents the expansion of cell clones with mutations. p53-deficiency would favor the expansion of cells containing damaged DNA, which can be oncogenic.

Three Ser/Thr kinases have been involved in sensing DNA damage, transducing signals, and activating p53. Double-strand breaks (DSB), one of the most severe types of DNA damage, activate the ATM, ATR, and DNA-PK kinases (Khanna and Jackson, 2001). ATM is a protein kinase that is deficient in ataxia-telangiectasia syndrome, a disorder characterized by radiosensitivity and genomic instability (Kastan et al., 1992). *atm*−/− cells are highly sensitive to ionizing radiation and show chromosomal instability and defects in multiple checkpoints of the cell cycle. Both p53 and Mdm2 can be phosphorylated by ATM kinase (Banin et al., 1998; Canman et al., 1998; Khosravi et al., 1999). Phosphorylation of p53 (Ser-15) and Mdm2 by ATM represses the Mdm2-mediated degradation of p53 (Maya et al., 2001). Other downstream targets of ATM include Brca1, Nbs1, Chk1, and Chk2 (Li et al., 2000; Cortez et al., 1999; Lim et al., 2000; Zhao et al., 2000). ATR is an ATM-related kinase, recognizing the same consensus sites, but showing little functional redundancy to ATM. ATR specifically mediates UV-induced rapid phosphorylation of p53 (Tibbetts et al., 1999). DNA-PK is a DSB repair enzyme that binds DSB, promotes nonhomologous end joining, and induces p53-dependent apoptosis but not growth arrest (Wang et al., 2000; Khanna and Jackson, 2001). In addition, Chk1 and Chk2 also phosphorylate p53 (Ser-20), which contributes to the stabilization and accumulation of p53 (Caspari, 2000; Chehab et al., 2000; Hirao et al., 2000).

In response to DNA damage, p53 activity is regulated through protein stabilization and posttranslational modification. Mdm2 targets p53 at the amino terminus and promotes the degradation of p53 through the ubiquitin-26S proteasome system (Kubbutat et al., 1997; Haupt et al., 1997). Since Mdm2 is transcriptionally activated by p53, the effects of p53 and Mdm2 on each other form a negative feedback control. p19ARF, one of the two products of INK4a/ARF locus, sequesters Mdm2 to the nucleoli and stabilizes p53 (Pomerantz et al., 1998; Zhang et al., 1998; Weber et al., 1999; Zhang and Xiong, 1999) (Fig. 14). DNA-damage-induced posttranslational modifications include at least phosphorylation, which has partially been discussed, and acetylation (Liu et al., 1999; Sakaguchi et al., 1998). In addition to the stabilization of p53, phosphorylation also enhances its transactivation activity and its association with cofactors (Lambert et al., 1998). The major phosphorylation sites in p53 have been mapped within the amino and carboxyl termini. p53 also is susceptible to acetylation at its lysine residues in the carboxyl terminal region. p300 and CBP, two coactivators for p53-mediated transactivation that physically interact with p53 (Avantaggiati et al., 1997;

Sang et al., 1997), and the recently identified p33ING2 are acetyltransferases and are able to acetylate p53 (Ito et al., 2001; Nagashima et al., 2001). However, the precise effects of such modification are still controversial (Gu and Roeder, 1997; Espinosa and Emerson, 2001).

Two p53 homologues have been identified and termed p63 and p73. p53, p63, and p73 form a structurally related protein family and share most of the features of p53 with differences in some aspects (Levrero et al., 2000; Irwin and Kaelin, 2001).

As discussed, p53 and pRb utilize different mechanisms to control different steps of cell division and to safeguard the reproduction of healthy daughter cells. In *RB*-deficient cells, deregulated DNA replication leads to p53 accumulation that eventually results in apoptosis. Although p53-induced apoptosis provides a way to actively remove *RB*-deficient cells, it is also construed to be a selective pressure for *RB−/−*, *p53−/−* cell. *RB−/−*, *p53−/−* cells completely lack the growth control of both p53 and pRb, rapidly accumulate more genomic alterations, and eventually form tumors. DNA tumor viruses typically use their oncoproteins to target both pRb and p53 in order to transform cells. As mentioned before, SV40 large T antigen targets p53 and pRb by direct interaction. Adenovirus uses E1A to target the pRb family and E1B oncoprotein to inactivate p53. HPV possesses E6 to accelerate the ubiquitination-mediated p53 degradation and E7 to disrupt pRb functions.

## Summary and Conclusion

Cell division is a very complex but well-coordinated process that involves several groups of genes. Proteins encoded by these genes play important roles in signal transduction, DNA replication, transcription regulation, protein synthesis, and degradation and morphogenesis. Deficiency in any part of the cell cycle machinery leads to growth arrest or apoptosis. Cyclins and Cdks are the essential forces that promote the cell cycle forward. pRb and p53 coordinately control the cell cycle at different steps. pRb is hypophosphorylated at G0 to early G1 and represses S phase gene expression by association with E2F and HDAC. Upon receiving the cell division signal, cyclin/Cdk complexes phosphorylate pRb, dissociate the pRb-HDAC-E2F complex, and thus activate E2F. E2F transactivates c-Myc and E2F itself. E2F and Myc transactivate a spectrum of genes that are required for DNA replication and S phase progression. At any phase during the cell cycle, DNA damage activates ATM/ATR kinases that will eventually activate p53. Enhanced p53 activity transcriptionally regulates target genes to induce growth arrest, DNA repair, or apoptosis to safeguard the integrity of the genome and faithful duplication of the cells. The transforming activity of DNA tumor virus oncoproteins depends on their capacity to inactivate both pRb and p53.

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CHAPTER

15

# MITOCHONDRIA AND OXIDATION IN THE REGULATION OF CELL DEATH

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Mitochondria have long been known to be critical for overall cell survival because of their role in energy metabolism. In the mid-1990s, however, it became evident that mitochondria also actively participate in a gene-regulated form of cell death known as apoptosis. Before the *epoch of apoptosis*, cell death was studied quite intensively in radiation biology. In searching for mechanisms of cell death in radio-sensitive tissues during the 1950s, a suppression of respiration—specifically between cytochromes b and c—in mitochondria from thymus and spleen was described (for a review, see Zhivotovsky et al., 1998). Moreover, the suppression of respiration was associated with the formation of pyknotic nuclei, now known to be a characteristic of cells undergoing apoptosis. In contrast, these phenomena were not observed in cells taken from radio-resistant tissues. Thus, the radiation lesion was believed to be due to impaired binding of cytochrome c to the mitochondrial inner membrane after X-irradiation (Scaife, 1964).

Complementary studies to those just described demonstrated that the addition of exogenous cytochrome c could stimulate respiration in mitochondria isolated from radio-sensitive, but not radio-resistant, tissues of irra-

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diated rats (Manoilov and Hanson, 1964). Importantly, loss of cytochrome c was not a result of its simple escape from mitochondria during the isolation procedure, since additional washing of the mitochondrial fraction with an isotonic buffer did not increase the "cytochrome c effect" (van Bekkum, 1957). Neither did in vitro irradiation of isolated mitochondria cause increased enzyme release. Thus, it was suggested that the perturbation of mitochondrial electron transfer in radio-sensitive tissues was based on a controlled release of cytochrome c from the mitochondria and the appearance of this protein in the cytosol.

Following relatively little movement over the next 25 years, Wang and coworkers demonstrated that the release of cytochrome c from mitochondria into the cytosol resulted in caspase activation and the execution of apoptosis (Liu et al., 1996). This critical observation, combined with the following important discoveries from other research groups, is largely responsible for a recent explosion in research focusing on mitochondrial regulation of apoptosis:

- The antiapoptotic protein Bcl-2 is localized in the outer mitochondrial membrane (Hockenberry et al., 1990).
- A membrane fraction containing mitochondria was required for the induction of nuclear apoptosis in a cell-free system (Newmeyer et al., 1994).
- A drop in mitochondrial transmembrane potential ($\Delta\Psi$) precedes chromatin cleavage and typical morphological changes induced by diverse apoptotic stimuli (Zamzami et al., 1995).
- Although reactive oxygen species (ROS) do not seem to be required for the execution step of apoptosis, they can be involved in the activation phase as intracellular signaling molecules (Jacobson, 1996).
- The apoptotic program can be halted at different stages by lowering intracellular ATP levels (Leist et al., 1997).

Taken together, a number of early observations and more recent findings have clearly demonstrated that mitochondria play an important role in cell death.

## Mitochondria: Central Components in the Activation of Apoptosis

The mitochondrion consists of two membranes: the inner and outer membranes (Fig. 1). The inner mitochondrial membrane, which forms the cristae, contains the molecular components of the electron transport chain and the ATP-synthase. Oxidation of mitochondrial respiratory chain substrates

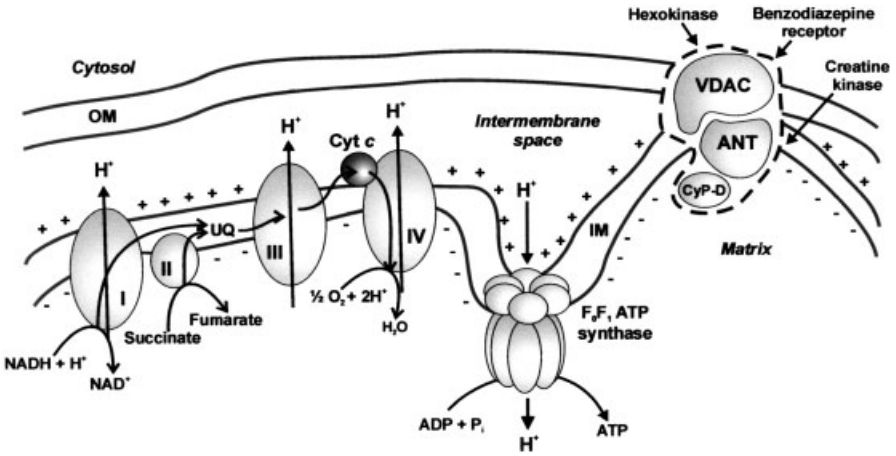


FIGURE 1. Schematic representation of the mitochondrial respiratory chain and the permeability transition pore.

results in the formation of a proton electrochemical gradient across the inner mitochondrial membrane. This gradient is exploited by the $F_0F_1$-ATP-synthase to produce ATP from ADP and $P_i$ (Fig. 1). Cytochrome c is a component of the electron transport chain and supports respiration by shuttling electrons between complex III (cytochrome bc1) and complex IV (cytochrome oxidase) (Fig. 1). Apo-cytochrome c is encoded by a nuclear gene and synthesized in the cytosol. It is imported into the mitochondrial intermembrane space in an unfolded configuration where it receives its heme group by heme ligase. Covalent attachment of this heme group stimulates a conformational change, and holo-cytochrome c subsequently assumes its functional role as a component of the electron transport chain. Importantly, cytochrome c is a basic protein, which is normally bound to the outer surface of the inner mitochondrial membrane (IMM) by an association with the acidic phospholipid cardiolipin, and evidence (see below) suggests that early modification of cardiolipin is important for cytochrome c release to occur during apoptosis (Fig. 2C).

Both in vitro and in vivo studies have demonstrated the release of cytochrome c from mitochondria during apoptosis, although the precise mechanism controlling this event is unclear (see below). Once in the cytosol, cytochrome c interacts with Apaf-1 (apoptosis protease activating factor) and pro-caspase-9, forming the apoptosome complex. The result is the cleavage and activation of pro-caspase-9 and other pro-caspases that are responsible for the execution stage of apoptotic cell death (Robertson et al., 2000). Importantly, only holo-cytochrome c, and not apo-cytochrome c, is able to stimulate pro-caspase-9 activation (Hampton et al., 1998). In fact, there is recent

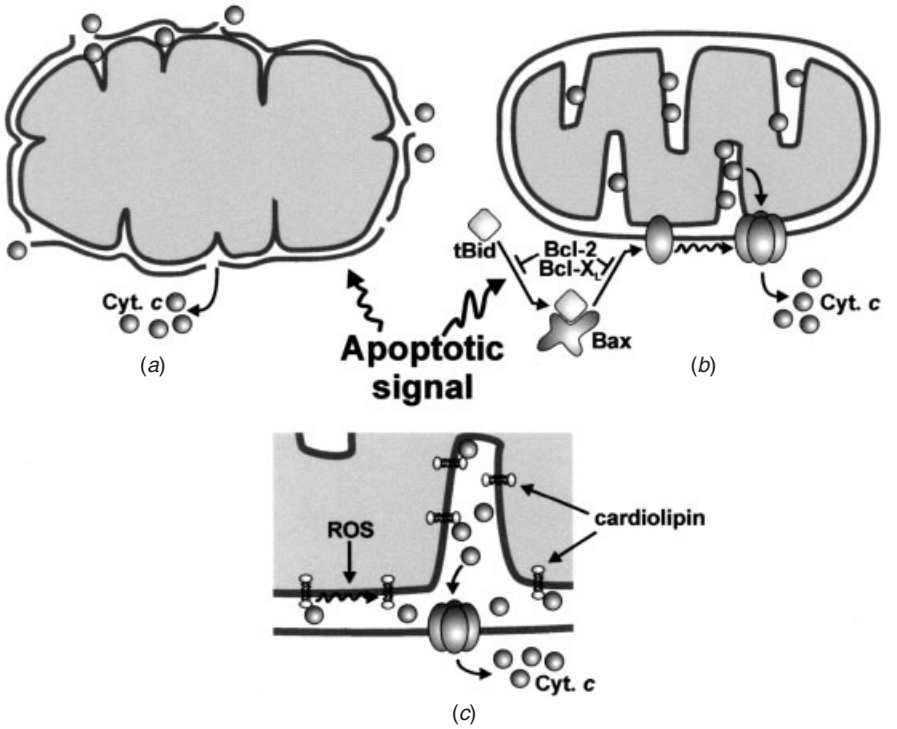


FIGURE 2. A schematic representation of mechanisms accounting for outer mitochondrial membrane (OMM) permeabilization and the release of cytochrome c. (A) Induction of permeability transition (PT) due to PT pore activation, leading to matrix expansion and rupture of the OMM. (B) Bax-mediated permeabilization of the OMM, involving tBid-induced Bax insertion and homooligomerization that can be inhibited by Bcl-2 or Bcl-$X_L$. (C) Peroxidation of cardiolipin is a key first step in mobilizing cytochrome c from the inner mitochondrial membrane prior to Bax-induced (B) permeabilization of the OMM.

evidence that apo-cytochrome c may inhibit apoptosome activity in the cytosol.

In addition to cytochrome c, several other proteins, normally located in the intermembrane space of mitochondria, are released into the cytosol during the early stages of apoptotic cell death. Among these proteins are Smac/DIABLO (second mitochondria-derived activator of caspase/direct IAP-binding protein with low pI), AK-2 (adenylate kinase), several procaspases, EndoG (endonuclease G), Omi/HtrA2, sulfite oxidase, HSPs (heat shock proteins), CIDE-B (cell-death-inducing DFF45-like effector), and ARTS (apoptosis-related protein in the TGF-β signaling pathway) (for a review, see van Loo et al., 2002). Another protein known as AIF (apoptosis-inducing

factor), whose intramitochondrial location is unclear, was also shown to be released from mitochondria during some forms of apoptosis.

Although the role of many of these proteins in the apoptotic process is well characterized, the involvement of others (AK-2, sulfite oxidase) is still unclear. In addition, it should be noted that the extra-mitochondrial function of many of these proteins is different from their specific role within mitochondria. For instance, mitochondrial cytochrome c is involved in supporting respiration, whereas once it is released into the cytosol, it acts as a cofactor for the activation of the caspase cascade. AIF is a flavin-adenine-dinucleotide-(FAD-)binding oxidoreductase, but neither its FAD-binding property nor its oxidoreductase activity is required for its ability to induce apoptosis after translocation to the nucleus (Susin et al., 1999). The functions of Smac/DIABLO, EndoG, and Omi/HtrA2 within mitochondria are also unclear. However, evidence suggests that once released from the mitochondria, Smac/DIABLO and Omi/HtrA2 are involved in the potentiation of caspase activity by relieving the caspase-inhibitory properties of the cytosolic inhibitor of apoptosis proteins (IAPs), whereas EndoG is translocated into the nucleus where it is involved in chromatin degradation (for a review, see van Loo et al., 2002).

The role of cytosolic caspases in the initiation and execution of cell death is well defined; however, the functions of pro-caspases that are constitutively located within mitochondria is still unclear. Recently, it was shown that the majority of mitochondrial caspase-3 and caspase-9 are S-nitrosated and thus undergo posttranslational inhibitory modifications (Mannick et al., 2001). During the apoptotic process, these caspases are denitrosated, allowing the catalytic site to function. However, it is unknown whether mitochondrial pro-caspases are activated within the mitochondria or must translocate to the cytosol in order to be activated.

The Bcl-2 family of proteins includes more than 20 members that fulfill pro- or antiapoptotic functions. All members of the Bcl-2 family contain up to four conserved domains, denoted Bcl-2 homology domains (BH1 to BH4) (for a review, see Cory and Adams, 2002). Accumulating evidence indicates that a number of Bcl-2 family proteins act, at least in part, at the level of mitochondria to either promote or prevent the release of apoptogenic proteins from the intermembrane space of mitochondria. In particular, Bcl-2 and Bcl-$X_L$, which can be found in the outer mitochondrial membrane and share 47% amino acid homology, block the release of cytochrome c and other proteins; however, the precise mechanism responsible for this effect is unclear. Reports describing the three-dimensional structure of Bcl-$X_L$ may reveal additional information about its function. Specifically, Bcl-$X_L$ protein possesses structural similarities with the pore-forming domains of bacterial toxins. Thus, it may be that Bcl-$X_L$'s capacity to form pores in lipid membranes is instrumental for its antiapoptotic function insofar as it maintains ion homeostasis across mitochondrial membranes. Additionally, it was demonstrated that

Bcl-2 and Bcl-$X_L$ may interact with the BH3 domain of pro-apoptotic Bcl-2 family molecules, namely, Bax, Bak, Bid, Bim, Noxa, and Puma. The ability to form heterodimers with these molecules was shown to be crucial for the antiapoptotic function of Bcl-2 or Bcl-$X_L$. However, in some experimental systems, mutants of Bcl-2 or Bcl-$X_L$ that do not interact with Bax retain significant antiapoptotic potency, suggesting that the antiapoptotic capacity of these proteins is only partially mediated by the functional sequestration of pro-apoptotic family members.

There is accumulating evidence that pro-apoptotic Bax, Bak, Bim, Puma, Noxa, and tBid proteins translocate to mitochondria in the presence of a death stimulus, where they exert their pro-apoptotic effect. Bax and Bak are reported to integrate into the mitochondrial membrane as oligomers in the presence of a pro-apoptotic stimulus. Recent evidence suggests that conformational changes in both proteins, or removal of 20 amino acids at the $NH_2$-terminal of Bax, are critical steps in their targeting to the outer mitochondrial membrane (OMM). Once integrated into the mitochondrial membrane, Bax and Bak are believed to elicit a pro-apoptotic response by stimulating the release of cytochrome c, an effect that is blocked by Bcl-2 or Bcl-$X_L$ in most systems. Like Bax and Bak, Bid exists in an inactive state prior to an apoptotic stimulus and undergoes posttranslational modification during its activation. Initial studies with Bid showed that an intact BH3 domain was essential for its ability to bind Bcl-2 or Bax and to promote cell death. This model was developed further by the understanding that the activation of Bid involves cleavage by caspase-8 and insertion of this product, tBid, into the outer mitochondrial membrane. Recently, other proteases, such as granzyme B, lysosomal proteases, and caspase-2, have been reported to also cleave Bid, resulting in an amplification of their pro-apoptotic effect by mitochondrial recruitment. Concerning caspase-2, there is also recent evidence that this protease can act directly on mitochondria, independently of Bid or other Bcl-2 proteins, to trigger cytochrome c release (Guo et al., 2002; Robertson et al., 2002). There is some controversy concerning the possible mechanism of Bim action. It is likely that Bim, by itself, does not induce release of cytochrome c; however, being a member of the BH3-only subfamily of proteins, it might bind and antagonize Bcl-2 or Bcl-$X_L$, as well as activate the BH1-3 proteins Bax and Bak (Terradillos et al., 2002).

## Multiple Routes and Mechanisms of Cytochrome c Release from Mitochondria

As mentioned above, cytochrome c is normally bound to the IMM by an association with the acidic phospholipid cardiolipin (Nicholls, 1974). Cardiolipin is unique to mitochondria and present predominantly, if not exclusively, in the IMM. Evidence suggests that dissociation of cytochrome c from

cardiolipin is a critical first step for cytochrome c release into the cytosol and the induction of apoptosis (Ott et al., 2002) (Fig. 2C). In particular, it was demonstrated that exposing submitochondrial particles to ROS produced by the mitochondrial electron transport chain stimulates a pronounced mobilization of cytochrome c and a concomitant loss of cardiolipin. Similarly, other studies showed that lowering mitochondrial cardiolipin content correlates not only with a decrease in respiration, but also with a stoichiometric increase in cytochrome c release. Further, a recent study from this laboratory demonstrated that simple permeabilization of the OMM by the addition of oligomeric Bax to isolated mitochondria in low ionic strength buffer is insufficient for cytochrome c release, and that peroxidation of cardiolipin may be a critical first step in order to mobilize cytochrome c from the IMM (Ott et al., 2002). Combined, these findings indicate that cardiolipin plays an important role in the structure and function of the respiratory chain, as well as in the retention of cytochrome c within the intermembrane space.

As already mentioned, the precise molecular mechanisms controlling cytochrome c release from mitochondria in the presence of a pro-apoptotic stimulus are not clear, although at least two distinct models for cytochrome c release have emerged that can be distinguished on the basis of whether $Ca^{2+}$ is required for the event. In one instance, mitochondrial $Ca^{2+}$ overload results in opening of a pore in the inner mitochondrial membrane with subsequent swelling, and the rupture of the outer membrane followed by the release of cytochrome c and other intermembrane space proteins (Crompton, 1999; Fig. 2A). Although it was originally believed that MPT (mitochondrial permeability transition) induction was the root mechanism responsible for cytochrome c release in response to different cytotoxic stimuli, more recently this notion has been challenged. Ample evidence from recent studies suggests that, although MPT is likely to be one mechanism responsible for cytochrome c release, it is no longer regarded as the *only* mechanism. In fact, it was shown that mitochondria of NGF-deprived sympathetic neurons undergoing apoptosis released cytochrome c and were reduced in size, yet remained intact and resumed normal function when reincubated with NGF (Martinou et al., 1999). A more recent study from our laboratory demonstrated that etoposide stimulated cytochrome c release from isolated mitochondria, despite the presence of 1 *mM* EGTA (a known inhibitor of MPT) in the reaction buffer. Thus, when $Ca^{2+}$ loading is insufficient to induce observable manifestations of MPT, the release of cytochrome c can still occur.

The $Ca^{2+}$-independent model asserts that a more selective protein release occurs without changes in mitochondrial volume (Fig. 2B). This mechanism involves specific channels or pores in the outer mitochondrial membrane that may be opened and regulated by certain pro-apoptotic members of the Bcl-2 family of proteins, including Bax. We reported recently that depend-

ing on experimental conditions, this protein can act either directly on mitochondria to stimulate the release of cytochrome c by forming a selective pore in the OMM, or it may facilitate opening of the PTP (permeability transition pore) (Gogvadze et al., 2001). A different study demonstrated the ability of recombinant Bax and cleaved or truncated Bid (tBid) to stimulate *complete* cytochrome c release that was unaffected by cyclosporin A and did not result in mitochondrial depolarization or alterations in ultrastructure as assessed by electron microscopy (von Ahsen et al., 2000). Recent evidence indicates that tBid induces a conformational change in Bax that allows this protein to insert in the outer membrane, oligomerize, and stimulate cytochrome c release (Eskes et al., 2000). These data pointed to the possibility that cytochrome c release involves a specific pore in the OMM that may be formed de novo by Bax or tBid.

The PTP consists of both inner and outer mitochondrial membrane proteins, such as ANT (adenine nucleotide translocator) and VDAC (voltage-dependent anion channel), respectively, and is formed at contact sites between these two membranes (Fig. 1). Recently, Tsujimoto's group demonstrated the ability of recombinant Bax, Bak, and Bim proteins to hasten the opening of VDAC in liposomes and induce changes in $\Delta\Psi$, whereas Bcl-$X_L$ was able to bind and close VDAC directly (Shimizu et al., 1999; Sugiyama et al., 2002). A subsequent and complementary study reported the ability of certain Bcl-2 family members, that is, Bid and Bik, to stimulate apoptosis in a fashion different from that of other pro-apoptotic Bcl-2 family proteins, such as Bax and Bak. Specifically, Bid and Bik stimulated cytochrome c release without inducing changes in $\Delta\Psi$ or interacting directly with VDAC. On the other hand, the same group has shown that Bim, which also belongs to the BH3-only subfamily, can activate VDAC directly. However, it is unclear how general this phenomenon is.

Hexokinases are known to bind to VDAC and directly couple intramitochondrial ATP synthesis to glucose metabolism. It was demonstrated recently that hexokinase II interferes with the ability of Bax to bind to mitochondria and induce the release of cytochrome c (Vander Heiden et al., 2001). Detachment of hexokinase from the mitochondria-enriched fraction isolated from HeLa cells promoted the binding of recombinant Bax-Δ19 and subsequent release of cytochrome c (Pastorino et al., 2002). Similarly, the addition of recombinant hexokinase II to the mitochondria-enriched fraction isolated from hepatocytes—cells that do not express this protein constitutively—prevented the ability of recombinant Bax-Δ19 to bind to mitochondria and promote cytochrome c release. Similar results were found in intact cells insofar as removing hexokinase II, or overexpressing it, resulted in a potentiation or inhibition, respectively, of Bax-induced mitochondrial dysfunction and cell death. Mitochondria-associated hexokinase activity is regulated by serine/threonine kinase Akt/PKB. It was suggested that Akt increases coupling of glucose metabolism to oxidative phosphorylation and regulates PTP

opening via the promotion of a hexokinase–VDAC interaction at the outer mitochondrial membrane (Gottlob et al., 2001).

ANT constitutes the most abundant protein of the mitochondrial inner membrane and catalyzes the import of cytosolic ADP and the export of matrix ATP synthesized during aerobic energy metabolism. Kroemer's group (Belzacq et al., 2002) has demonstrated that a direct physical interaction occurs among ANT, Bax, and Bcl-2. They found that ANT-Bax channels were selectively permeable to cations, whereas Bax channels were selectively permeable to anions. Importantly, Bcl-2, bongkrekic acid (a specific ANT inhibitor), or ATP and ADP (the natural ligands of ANT) closed ANT-Bax channels. Functional analysis of ANT pore opening in liposomes demonstrated that ANT might be a target for multiple apoptosis modulators. It seems that ANT is a bifunctional protein: a vital ADP/ATP translocator, and a lethal pore regulator. However, the nature of the signal and how it can induce a switch from one ANT function to another are unclear. Moreover, it is unclear how ANT may integrate death signals arising from so many different stimuli.

Finally, recent studies employing patch-clamping of intact mitochondria and proteoliposomes generated from these organelles have uncovered a novel ion channel whose activity correlates with the onset of apoptosis (Pavlov et al., 2001). The pore or channel diameter, inferred from the largest conductance state of this channel, is sufficient to allow the diffusion of cytochrome c and larger proteins. The activity of the channel is affected by Bcl-2 family proteins in a manner that is consistent with their pro- or antiapoptotic properties. A similar channel activity was found in OMMs isolated from yeast expressing human Bax. These findings implicate this mitochondrial apoptosis-induced channel as a candidate for the outer membrane pore through which cytochrome c and other factors exit during apoptosis. However, it is unclear how the activity of this channel relates to other documented release mechanisms for intermembrane space proteins.

It is still unclear whether cytochrome c and other mitochondrial proteins are released simultaneously and by the same mechanism. In fact, although Bcl-2 blocks the release of cytochrome c and Smac/DIABLO equally well, Smac/DIABLO release has been found to be caspase-dependent, which is normally not true for cytochrome c release (Adrain et al., 2001). A more recent study examined cytochrome c and Smac/DIABLO release at the single cell level and found that cytochrome c release preceded that of Smac/DIABLO (Springs et al., 2002). Although the reason for this is unclear, it is possible that release of these proteins is affected by differences in their intramitochondrial localization or by mitochondrial remodeling, especially that of cristae (Scorrano et al., 2002).

## Role of Oxidative Stress in the Modulation of Apoptosis

The role of oxidative stress in apoptosis has been shaped by several independent observations. Thus, direct treatment of cells with oxidants, such as hydrogen peroxide or redox-active quinones, was originally thought to exclusively cause necrosis, but more recent studies have shown that lower concentrations of these agents can trigger apoptosis (Hampton and Orrenius, 1997). In addition, many groups have suggested that the generation of intracellular ROS (reactive oxygen species) may constitute a conserved apoptotic event, and cite ROS production as a critical determinant of toxicity associated with exposure to ionizing radiation and chemotherapeutic drugs. Depletion of glutathione (GSH) pools has also been suggested to be part of the cell death effector machinery since it often accompanies ROS production (Macho et al., 1997). Moreover, oxidative modification of proteins and lipids has been observed in cells undergoing apoptosis, also in response to non-oxidative stimuli, suggesting that intracellular oxidation may be a general feature of the effector phase of apoptosis. The ability of various cellular antioxidants to block apoptosis induced by diverse agents other than oxidants also argues for the central role of oxidative stress in apoptosis. Reciprocally, broad-spectrum antiapoptotic proteins like Bcl-2 and the baculovirus protein p35 have been ascribed an antioxidant function, indicating that ROS generation may be a requisite apoptotic event.

Several mechanisms for ROS induction of apoptosis have been proposed; however, an integrated model has yet to be established. We generally favor a scheme in which ROS, for example, $H_2O_2$, act upon mitochondria, causing a disruption of mitochondrial membrane potential and the release of cytochrome c; this, in turn, is followed by the activation of caspase cascade (Stridh et al., 1998). An alternative model for ROS-induced apoptosis involves upregulation of the Fas/FasL system (Dumont et al., 1999). The observation that various chemotherapeutic drugs cause intracellular ROS production and Fas upregulation has perpetuated this paradigm; however, other studies contend that $H_2O_2$-induced apoptosis is Fas-independent. Finally, transcription factors can be modulated by oxidative stress. Nuclear translocation of p53 and the ubiquitous transcription factors, NFκB and AP-1, are activated by ROS (Pinkus et al., 1996). Once activated, these transcription factors might drive transcription of pro-apoptotic genes or perhaps cause expression of inhibitors of survival-related proteins. The issue of p53-induced apoptosis has gained momentum in the oxidative stress field recently since the activation of p53 has been shown to increase the synthesis of proteins that generate or respond to oxidative stress. Taken together, these data suggest that some of the above-mentioned mechanisms for ROS-induced apoptosis may be linked.

TABLE 1. Components of the Apoptotic Pathways Modified by Oxidative Stress

| | |
|---|---|
| Plasma membrane | Fas |
| | Phosphatidylserine |
| Cytosol | Caspase-3 |
| | Caspase-8 |
| | Caspase-9 |
| Mitochondrion | Cardiolipin |
| | Caspase-3 |
| | ANT |
| | $\Delta\Psi$ |
| Nucleus | Transcription factors (NFκB, AP-1, p53) |

## Components of the Apoptotic Machinery that May be Modulated by Oxidative Stress

Several components of the apoptotic machinery are susceptible to modulation by oxidants (Table 1). Thus, mitochondrial regulation of apoptosis, coupled with this organelle's innate production of ROS, makes it a likely candidate for such modulation. The release of cytochrome c from mitochondria, which was described above, is critical for the mitochondria-dependent pathway of caspase activation and is an important amplification step for other models of caspase activation, such as death receptor ligation or granzyme B. Despite its apparent indispensability for several models of apoptosis, the precise mechanism by which cytochrome c is released remains contentious (Martinou and Green, 2001; Zamzami and Kroemer, 2001). As was mentioned above, existing models for cytochrome c release can be divided roughly into $Ca^{2+}$-dependent or -independent paradigms. Increases in cytosolic $Ca^{2+}$, oxidants, and inorganic phosphates are all classical triggers of MPT, while pore opening is blocked by cyclosporin A and bongkrekic acid. Once triggered, MPT results in mitochondrial swelling, outer membrane rupture, and release of mitochondrial proteins. Interestingly, the ability of cytochrome c to be released may rely significantly on the peroxidation status of specific mitochondrial elements. As mentioned, under normal conditions, the bulk of cytochrome c is tethered to cardiolipin, a phospholipid, which is a unique component of the mitochondrial inner membrane. In order for cytochrome c release to occur, it must first be released from the grip of cardiolipin before it can exit the mitochondria (Ott et al., 2002).

Several independent groups have reported alterations in cardiolipin that occur concomitantly with cytochrome c release. Peroxidation of cardiolipin initiates the release of cytochrome c from mitochondria by dissociating cytochrome c from the mitochondrial membrane (Shidoji et al., 1999). In vitro

experiments using cytochrome c, preferentially bound to cardiolipin liposomes, have demonstrated that cytochrome c is released from liposomes by oxidation of cardiolipin. An antioxidant protein, phospholipid hydroperoxide glutathione peroxidase (PHGPx), has been shown to inhibit apoptosis and prevent cytochrome c release by a number of triggers; it also prevents cardiolipin peroxidation. Overexpression of mitochondrial PHGPx also inhibits the opening of the mitochondrial permeability transition pore by oxidative stimuli (Nomura et al., 2000). In light of these findings and what was discussed earlier, the modification of cardiolipin may be an obligatory step in initiating the liberation and release of cytochrome c from mitochondria. This is further supported by the finding that tBid inserts preferentially into mitochondria at sites where cardiolipin is present, and thereby facilitates the release of cytochrome c. Thus, cardiolipin serves as a docking site for tBid (Lutter et al., 2000). Whether this holds true for other pro-apoptotic Bcl-2 family members remains to be seen.

Caspases contain an active-site cysteine nucleophile, which is prone to oxidation or thiol alkylation. It is therefore not surprising that the activity of caspases is optimal under reducing environments. Any deviation from such reducing conditions within an injured cell could be detrimental to caspases and render them inactive. It has been demonstrated by our group that $H_2O_2$ suppresses both the activation and activity of caspases, possibly through modulation of the redox status of the cell and the oxidation of cysteine residues in caspases. Furthermore, the oxidation of dithiocarbamates to thiuram disulfides has been shown to inhibit apoptosis through disulfide formation at the active-site cysteine of caspases, leading to an inhibition of their enzymatic activity (Nobel et al., 1997).

Exposure to NO or activation of iNOS has also been reported to inhibit apoptosis in several cell types (Melino et al., 1997). NO-mediated inhibition of apoptosis in most cases is due to direct inhibition of caspase activity through S-nitrosation of the active-site cysteine conserved in all caspases. NO inhibition of caspases is reversible by dithiothreitol, consistent with direct S-nitrosation of the caspase catalytic cysteine residue. Interestingly, pro-caspase-3 was recently shown to be S-nitrosated on its catalytic site cysteine (Cys-163) in unstimulated human cell lines, and was denitrosated upon activation through the Fas pathway. It was proposed that nitrosation/denitrosation might serve as a regulatory mechanism, analogous to phosphorylation/dephosphorylation, during apoptotic cell death. Indirect effects of NO on caspases can also be a component of toxicity in certain systems.

Work carried out in our laboratory has demonstrated that the redox-active quinone, menadione, could not only induce necrosis in HepG2 cells, but that exposure of Fas-treated cells to menadione also switched the mode of cell death to necrosis. Menadione, like other redox-active quinones, is known to have cytotoxic effects, which are mediated through oxidative stress and alterations in cellular $Ca^{2+}$ homeostasis. Metabolism of menadione leads

to the generation of ROS and to the oxidation of GSH to GSSG. Preincubation of apoptotic cells with catalase reduced the ROS levels and reversed the inhibitory effect of menadione on caspase activity. Taken together, these results strongly suggest that the inhibitory effects of menadione are due to the production of hydrogen peroxide and subsequent inactivation of caspases, and not to the direct effect of menadione on caspase thiol group(s) (Samali et al., 1999).

An important feature of apoptotic cell death is efficient clearance of dying cells by macrophages, and appearance of the aminophospholipid, phosphatidylserine (PS), on the outer leaflet of the cell membrane serves as a recognition signal for macrophages to engulf the apoptotic cell. Both PS exposure and phagocytosis appear to be events that can be modulated by oxidative stress. Diamide and N-ethylmaleimide (NEM) are two classic examples of thiol-reactive compounds that are capable of creating oxidative stress. Both compounds efficiently lower intracellular thiol levels, resulting in GSH depletion. However, these agents may also cause MPT, probably by modifying critical sulfur residue(s) in this complex. Whether the mitochondrial dysfunction precedes the GSH depletion seen with these agents is not known (Fadeel et al., 1999). Interestingly, it has been demonstrated that NEM and diamide can cause PS exposure. One of the enzymes that maintain normal phospholipid asymmetry, the aminophospholipid translocase, has been shown to contain a critical thiol residue, which may be modified by NEM or diamide treatment (Waring et al., 1999). However, it is unclear whether PS exposure by these agents is due to an inhibitory effect on the aminophospholipid translocase, or if the mitochondrial or GSH-related effects of these agents result in PS exposure by some other mechanism.

Observations indicating that several cytoprotective proteins can also defend cells against oxidative stress have bolstered the role of redox regulation in homeostasis. One of the most widely recognized antiapoptotic proteins is Bcl-2 (see above). This protein resides in mitochondrial, ER, and nuclear membranes, and for each of these subcellular localizations, a different protective mechanism has been proposed. It has also been suggested that Bcl-2 inhibits cell death by diminishing the generation of reactive oxidants, thus preventing critical intracellular oxidations that are requisite for the completion of the apoptotic program. Separate studies of Bcl-2-overexpressing cells illustrate that they have higher levels of total cellular glutathione. Expression of Bcl-2 has also been shown to increase intracellular GSH by inhibiting methionine-dependent GSH efflux, implicating the sinusoidal GSH transporter in the protective effects of Bcl-2 (Meredith et al., 1998). Bcl-$X_L$ is another antiapoptotic member of the Bcl-2 family of proteins, which may exert its effects, at least in part, through GSH modulation. In an IL-3-dependent murine pro-lymphocytic cell line, Bcl-$X_L$ overexpression has been shown to prevent drops in GSH and to block apoptosis following IL-3 withdrawal (Bojes et al., 1997).

## Role of Antioxidants in the Protection against Apoptosis

The tripeptide GSH is the most abundant weapon against intracellular ROS accumulation and regulates the redox state of many other cellular components. Drops in GSH levels and concomitant increases in ROS during the apoptotic process have been reported by several groups. In Fas-treated Jurkat cells, the drop in GSH was shown not to be due to an inhibition of GSH synthesis, or oxidation of GSH to GSSG, nor to a deficit in the GSH salvage pathway, but to an increased rate of GSH efflux (van den Dobbelsteen et al., 1996). This was ascertained by quantitatively recovering the reduced form of the GSH from the medium. The efflux of GSH was blocked by several inhibitors of the canalicular membrane GSH transporter, including bathophenanthroline disulfonate (BPS) and bathocuproine disulfonate (BCPS) (Oda et al., 1999).

Redistribution of cellular GSH is another event that may be critical during apoptosis. Although GSH is synthesized in the cytosol, it is transported into organelles, including mitochondria and the nucleus, where it can be used as a cofactor in glutathione peroxidase- and S-transferase-mediated reactions. Nuclear pools of glutathione appear to be more resistant to depletion by agents like buthionine sulfoximine (BSO), diethyl maleate (DEM), and NEM than cytosolic stores of GSH, suggesting that GSH is an important guardian against oxidative damage to DNA and nuclear proteins (Bellomo et al., 1992). More recently, overexpression of the antiapoptotic protein Bcl-2 was found to promote sequestration of GSH into the nucleus, indicating that suppression of apoptosis may be linked to modulation of the nuclear redox state by GSH (Voehringer et al., 1998). However, it has also been suggested that the impact of Bcl-2 on glutathione metabolism is cell-line-dependent. These data addressed GSH accumulation and consumption upon treatment with N-acetylcysteine (NAC) and neocarzinostatin (NCS), respectively, and did not address compartmentalization in the overexpressing cells. Interestingly, 10 to 15% of the total level of intracellular GSH is localized to mitochondria, and depletion of mitochondrial GSH causes increased sensitivity to killing by antimycin A, which stimulates mitochondrial ROS production by inhibiting electron transport. Whether drops in GSH levels precede intracellular ROS production during apoptosis has been difficult to discern. However, treatment with BSO does not cause apoptosis in many cell types, indicating that GSH depletion alone may not trigger apoptosis (Schor et al., 2000). In addition to the glutathione system, superoxide dismutases (SODs) are critical components of the cell's natural antioxidant defenses. These enzymes constitute the first line of defense against oxygen toxicity and exist as copper/zinc (Cu, Zn-SOD) and manganese (Mn-SOD) metalloproteins in mammalian tissues. Mn-SOD is localized in the mitochondrial matrix, whereas Cu, Zn-SODs function in the cytosol as well as in the

extracellular space (Ec-SOD). Interestingly, insertion of the cytosolic enzyme into mitochondria can protect cells lacking Mn-SOD from oxidative stress-induced apoptosis, suggesting that localization precedes differences between the SOD species. Although SODs efficiently reduce superoxide, they also generate hydrogen peroxide as a byproduct of this function, which can be detrimental.

## Mitochondrial Involvement during Cell Death in Worms and Flies

Strikingly, many of the apoptotic components that are involved in the mammalian mitochondrial pathway are evolutionarily conserved and critically involved in cell death also in the worm and fly (Fig. 3). In the nematode *C. elegans*, for instance, CED-4 (ced, meaning cell death abnormal), an analogue of mammalian Apaf-1, is normally associated with mitochondria but translocates and assumes a perinuclear location during apoptosis. Importantly, CED-4 translocation precedes the activation of CED-3, just as Apaf-1 activity precedes that of the CED-3 mammalian homologue, caspase-3. Thus, although the molecular mechanisms and significance of CED-4 translocation are still unknown, a disruption of this protein's normal association with the mitochondria may act to drive the cell death process in the worm (Chen et al., 2000). As discussed above, cytochrome c release is important for the

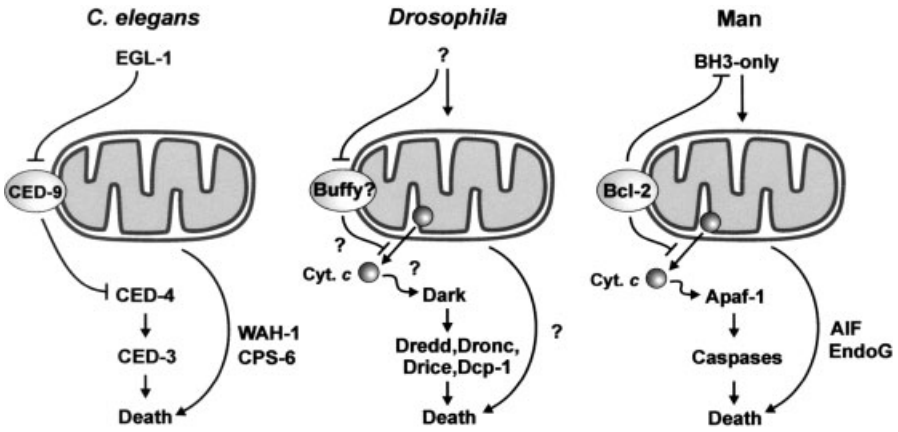


FIGURE 3. Conservation of core death genes among *C. elegans*, *Drosophila*, and man. Although mitochondria, in general, have been important cell death regulators throughout evolution, a key difference is that mitochondrial cytochrome c release is required for Apaf-1 activation in man, whereas cytochrome c release is not required for CED-4 activation in *C. elegans*. Whether cytochrome c is required for Dark activation in *Drosophila* is unclear.

activation of Apaf-1 via an interaction with a WD-40 repeat domain found at the C-terminus of Apaf-1. In contrast, cytochrome c is not required for the activation of CED-4, which lacks a similar WD-40 repeat, and cytochrome c is not released from mitochondria during cell death in the nematode (Metzstein et al., 1998).

In *Drosophila*, the Apaf-1/CED-4 homologue is known as DARK (*Drosophila melanogaster* Apaf-1-related killer) and is essential for apoptosis induced by diverse stimuli. Structurally, DARK is more similar to Apaf-1 than Ced-4. Although in vitro binding between DARK and cytochrome c has been demonstrated, just as in the worm, cytochrome c is not released from *Drosophila* mitochondria during stress-induced apoptosis and thus is not available for such binding (Zimmermann et al., 2002). In fact, the precise mechanism for DARK activation is unknown; however, it seems that its activation precedes the activation of the caspase cascade, which is consistent with mammalian and *C. elegans* models (Dorstyn et al., 2002). Of interest is the fact that a significant proportion of the *Drosophila* initiator caspase DRONC and effector caspase DRICE appears to localize near mitochondria, suggesting that mitochondria may play some role in caspase activation also in the fly.

As mentioned above, AIF and EndoG belong to the group of pro-apoptotic mitochondrial proteins that are released into the cytosol during apoptosis (for a review, see van Loo et al., 2002). After translocation to the nucleus, they exert their apoptogenic functions by mediating caspase-independent DNA degradation. Exactly how this occurs is unclear, since AIF has no apparent nuclease activity and high concentrations of EndoG are required to induce DNA degradation in vitro. Thus, it appears that these two proteins do not act in isolation but must interact with each other, or with other proteins, to achieve full activity.

Support of such a notion was provided recently when homologues of AIF (WAH-1) and EndoG (CPS-6) were discovered in *C. elegans* (Wang et al., 2002). Similar to mammalian systems, WAH-1 and CPS-6 are normally present in the mitochondria of the nematode. During cell death, these proteins are released from mitochondria and translocate to the nucleus to induce DNA degradation. In doing so, it appears that WAH-1 specifically interacts with CPS-6, and as a result of this association, both mitochondrial proteins cooperate to promote DNA fragmentation. Thus, it appears that AIF and EndoG represent a unique mitochondrial pathway leading to apoptotic DNA degradation that is conserved between *C. elegans* and mammals.

## Concluding Remarks

Many lines of research have focused recently on achieving an emergent understanding of the role mitochondria play in the regulation of cell death.

Despite researchers' best efforts, however, mitochondrial involvement in apoptosis remains one of the more contentious issues in the field. Still, very few investigators would challenge the importance of these organelles as critical modulators of apoptosis. Additionally, emerging evidence indicates that different steps in the apoptotic pathway, including cytochrome c release and caspase activity, are often regulated by changes in the intracellular redox state, which may serve as a "switch" between apoptosis and necrosis. Combined, however, it is clear from the present discussion that a number of issues relating to the precise role of mitochondria and oxidation in cell death are unresolved and require further study.

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SECTION

# IV

# Deregulation of Cell Death in Disease and Future Intervention

CHAPTER

16

# THE USE OF PROTEOMICS TO IDENTIFY AND CHARACTERIZE CELL DEATH PROTEINS

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## ABBREVIATIONS

| | |
|---|---|
| Ac | Acetyl |
| AIF | Apoptosis-inducing factor |
| AK2 | Adenylate kinase 2 |
| Apaf-1 | Apoptotic protease-activating factor 1 |
| ASC | Apoptosis-associated specklike protein containing a CARD |
| ATP | Adenosine triphosphate |
| Bax | Bcl2-associated X protein |
| Bbc3 | Bcl-2-binding component 3 |
| Bcl-2 | B-cell lymphoma 2 |
| BH | Bcl-2 homology domain |
| Bid | BH3-interacting death agonist |
| Bim | Bcl-2-interacting mediator of cell death |
| BIR | Baculovirus inhibition of apoptosis protein repeat |
| BNIP3 | Bcl-2/adenovirus E1B 19 kD-interacting protein 3 |
| BNIP3L | Bcl-2/adenovirus E1B 19 kD-interacting protein Like |
| CAP | Cytotoxicity-dependent APO-1-associated protein |

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| | |
|---|---|
| CARD | Caspase-activating recruitment domain |
| caspase | Cysteinyl aspartate-specific protease |
| CD95 | Cluster of differentiation antigen 95 |
| cDNA | Complementary DNA |
| Ced-3 | Cell death defective-3 |
| Ced-4 | Cell death defective-4 |
| Ced-9 | Cell death defective-9 |
| CID | Collision-induced dissociation |
| CPP32 | Cysteine protease protein of 32 kDa |
| dATP | Deoxyadenosine triphosphate |
| DCP-1 | *Drosophila* caspase-1 |
| DD | Death domain |
| 1, 2-DE | One- or two-dimensional gel electrophoresis |
| DED | Death effector domain |
| DIABLO | Direct IAP-binding protein with low pI |
| DISC | Death-inducing signaling complex |
| DP5 | Death-promoting factor 5 |
| DR5 | Death receptor 5 |
| DREDD | Death-related *ce*d-3/*Ne*dd2-like gene |
| egl-1 | Egg-laying defective 1 |
| ES | Electrospray |
| ESI | Electrospray ionization |
| EST | Expressed sequence tag |
| FADD | Fas-receptor-associated death domain |
| FLICE | FADD-like interleukin-1β-converting enzyme |
| FMK | Fluoromethylketone |
| Gαq | Alpha component of the heterotrimeric Gq protein |
| GST | Glutathion-S-transferase |
| HRK | Harakiri |
| HSN | Hermaphrodite specific neurons |
| Htra | High-temperature requirement |
| IAP | Inhibition of apoptosis protein |
| ICE | Interleukin-1β-converting enzyme |
| IL-1β | Interleukin-1β |
| IRES | Internal ribosome entry site |
| IRF-1 | Interferon responsive factor 1 |
| MACH | Mort1-associated Ced-3 homolog |
| MALDI | Matrix-assisted laser desorption/ionization |
| MEF | Mouse embryonic fibroblast |
| MORT | Mediator of receptor-induced toxicity |
| mRNA | Messenger ribonucleic acid |
| MS | Mass spectrometry |
| MS/MS | Tandem mass spectrometry |
| NIP3L | Adenovirus E1B 19 kD-interacting protein 3 Like |

| | |
|---|---|
| Nix | Nip3-like protein X |
| NOD | Nucleotide-binding oligomerization domain |
| PCR | Polymerase chain reaction |
| PSD | Postsource decay |
| PUMA | p53-upregulated modulator of apoptosis |
| PYCARD | Pyrin- and CARD-containing protein |
| RDA | Representational difference analysis |
| RIP | Receptor interacting protein |
| RP | Reverse phase |
| RPA | RNase protection assay |
| SAGE | Serial analysis of gene expression |
| SDS | Sodium dodecyl sulphate |
| Smac | Second mitochondria-derived activator of caspase |
| TAP | Tandem affinity tagged |
| tBid | Truncated Bid |
| TGFβ | Transforming growth factor β |
| TNF-R1 | Tumor necrosis factor-receptor 1 |
| TOF | Time of flight |
| TRAIL | Tumor-necrosis-factor-related apoptosis-inducing ligand |
| TRAIL-R2 | TRAIL-receptor 2 |
| XIAP | X-linked inhibitor of apoptosis protein |
| Y2H | Yeast two-hybrid |
| z | Benzyloxycarbonyl |

Today's genome sequencing and differential messenger RNA expression analysis techniques have generated an immense amount of data, providing the opportunity for cell biologists to investigate molecular signaling pathways at a much larger scale than previously anticipated. In addition, the technology platform to analyze and try to understand the function of proteins, namely, proteomics, in complex biological samples has advanced steadily. This chapter summarizes some contributions of these rapidly evolving technologies in the field of cell death research (overview in Table 1). In the first section of this chapter, "Genomics in Cell Death," we illustrate how the conserved domain architecture of cell death proteins and the access to an increasing number of genomic sequences have led to the comprehension that the basic cell death machinery is present in all kingdoms of life. "Transcriptomics in Cell Death," the second part of this chapter, presents different examples from the literature in which transcriptional profiling techniques have advanced our understanding of cell death pathways. "Proteomics," the third section, comprises an introduction to the technology of mass spectrometry to identify proteins in complex mixtures, and a glimpse into gel-free proteomics. The subsequent discussion on proteomics in cell death research highlights the use of yeast two-hybrid, affinity-based, and gel filtration techniques, and mass spectrometry to analyze cell death signaling

TABLE 1. Overview of the Technologies Used to Identify Cell Death Proteins

| Approach | Methodology | Example | Reference |
|---|---|---|---|
| Genomics | Homology search, mutational studies | Identification of para- and metacaspases | Uren et al. (2000) |
| Transcriptomics | Arrays, SAGE, differential display, RDA, RPA, EST profiling | Nix upregulation in hypertrophic heart | Yussman et al. (2002) |
| Proteomics | | | |
| Interaction study | Y2H, affinity capture, MS | Death domain receptor signaling | Kischkel et al. (1995) |
| Complex isolation | Gel filtration, density gradient, MS | Apoptosome isolation | Cain et al. (1999) |
| Differential protein profiling | Subcellular fractionation, 1- and 2-DE, MS | Mitochondrial apoptogenic molecules | van Loo et al. (2002a) |
| Cell free reconstitution | Subcellular fractionation, activity readout, MS | Cytochrome c/dATP activate caspases | Liu et al. (1996) |
| Activity profiling | Mechanism-/affinity-based<br>Immobilization<br>Substrate chip, FRET, MS | Quantitative caspase-3 activity profiling | Winssinger et al. (2002) |

complexes. The last part of this chapter focuses on the development of novel, protease-activity-based approaches in a nonhypothesis-driven way, revealing unexpected modulatory pathways in cell death.

## Genomics in Cell Death

The ultimate biochemical features of apoptosis such as cytoplasmic shrinkage, chromatin condensation and fragmentation, membrane blebbing, and phosphatidyl serine exposure rely on the existence of a limited number of conserved protein domains present in pro- and antiapoptotic proteins. These domains include the death domain (DD), the caspase recruitment domain (CARD), the death effector domain (DED), the pyrin domain, the Bcl-2 homology domain (BH), the nucleotide-binding oligomerization domain (NOD), and the baculovirus IAP repeat (BIR) domains (Aravind et al., 1999, 2001; Inohara and Nunez, 2001; Uren et al., 2000; Verhagen et al., 2001). During evolution these domains emerged as "building blocks" that became arranged in distinct linear combinations, often together with other protein functions (e.g., kinases and proteases), to give rise to a plethora of gene products operating in apoptotic signal transduction and execution and far beyond (Aravind et al., 2001). The availability of an increasing number of genome sequences allows the systematic search for the presence of paralogs (significantly similar counterparts present in the same organism) and orthologs (direct evolutionary counterparts present in a different organism) among the proteins encoded in these genomes. Phylogenetic analysis of the theoretical protein set of *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Homo sapiens* on the presence and arrangement of archetype domains from known apoptotic proteins has revealed the increasing degree of complexity of the apoptotic molecular machinery in vertebrates relative to insects and nematodes (Aravind et al., 2001). Furthermore, such analysis, supported by a comparison of structural data, has demonstrated that a considerable number of apoptosis-associated domains or structural motifs are even present in bacteria, fungi, and plants (Aravind et al., 1999; Uren et al., 2000). A notable example of this comparative genomics approach is the identification of two families of caspaselike proteins, namely, the paracaspases found in metazoans and *Dictyostelium* and the metacaspases found in plants, fungi, and protozoa (Uren et al., 2000).

Clearly, genomic data analysis provides a wealth of information to cell death research, but, as with any other scientific approach, it also has its limitations. For example, the Bcl-2 protein family is conserved in animals but in particular the BH3-only subfamily displays hardly any significant mutual sequence similarity, and therefore is not amenable to genome-wide comparison studies. In addition, in more complex organisms, molecules emerged with clear homology to core apoptotic proteins, yet displaying additional

functions that are, according to current knowledge, unrelated to apoptosis. Survivin, for example, has a role in cell cycle regulation, yet its BIR domain architecture advocates its classification in the IAP family (Ambrosini et al., 1998). The CARD-containing proteins represent another example of diversification with members functional in inflammation and immune responses such as caspase-1, RIP2, NOD1 and NOD2, Bcl-10, CARD-10, and CARD-11 (Bouchier-Hayes and Martin, 2002). Furthermore, activation of pro-apoptotic caspases does not always lead to cell death. Caspase-3, -6 and -8 activity is even required for T lymphocyte activation and proliferation (Alam et al., 1999; Kennedy et al., 1999; Miossec et al., 1997). Although genome comparisons provoke many questions, they provide leads for designing experiments that will eventually allow the functional annotation of the genetic information with respect to cell death. The functional genetic approaches that are applicable to model organisms such as *Saccharomyces cerevisiae*, *C. elegans*, and *D. melanogaster* will surely assist in providing some of the needed answers. The availability of complete genome sequences allows the prediction of all putative proteins encoded in a genome (defined as the proteome). This information has also facilitated the use of mass spectrometry (MS) to identify proteins: The sequence of a protein does not need to be determined de novo, but rather the task is to recognize the determined peptide sequence or peptide fingerprint in available databases. These databases are based in part on predicted protein sequences and, provided that the genome annotation covers the complete proteome of a given organism, this information strongly limits the number of possible peptide sequences and proteolytic fingerprints that can be obtained from a given organism. Therefore, even a limited number of peptide masses after proteolytic digestion of proteins will usually allow the correlation of a characteristic "peptide mass fingerprint" with a unique protein (see below).

## Transcriptomics in Cell Death

With the advent of cDNA and oligonucleotide arrays, it became possible to simultaneously monitor the messenger RNA expression profile of thousands of genes, the so-called transcriptome (Shoemaker and Linsley, 2002). Without a doubt, such immense information output is an asset of gene array technology. At the same time, the overflow of data often makes it difficult to deduce adequate information and to distinguish between causative and circumstantial changes in expression profiles. Here we attempt to highlight a number of examples where transcriptional profiling techniques have advanced our understanding of cell death pathways. We focus on BH3-only proteins.

Connections between certain stress conditions such as DNA-damaging agents and transcriptional induction of pro-apoptotic genes such as Bax

(Schmidt et al., 1999), Apaf-1, and Fas ligand have been reported (Kannan et al., 2001; Kasibhatla et al., 1998; Miyashita and Reed, 1995; Robles et al., 2001). These upregulations are often, but not exclusively, dependent on the activation of the tumor suppressor protein p53. P53 controls apoptosis, at least partially, by transcriptional activation of target genes (Shen and White, 2001). Differential transcription profiling approaches in response to p53 activation have led to the discovery of several novel BH3-only Bcl-2 proteins. The differential display method is a method comparing mRNA expression levels in different experimental settings. The method is based on the comparison among different samples of the amount of cDNA fragments generated by reverse transcription and subsequent amplification using a set of random decamer forward primers and oligodTXY (X = A, C, or G; Y = A, C, G, or T) reverse primers (Liang and Pardee, 1992). With this technique, Noxa has been identified as a gene that was induced upon X-ray irradiation of wild-type but not interferon responsive factor 1 (IRF-1)/p53 double-deficient MEFs (Oda et al., 2000). Noxa encodes a 103 (mouse) or 54 (human) amino acid residue long BH3-only protein that is transcriptionally upregulated by p53 to stimulate the mitochondrial apoptotic pathway. Likewise, PUMA (p53-upregulated modulator of apoptosis) was cloned after microarray analysis (Butte, 2002) and serial analysis of gene expression (SAGE) (Velculescu et al., 1995) of transcripts upregulated upon p53 gene expression (Nakano and Vousden, 2001; Velculescu et al., 1995; Yu et al., 2001). PUMA, dubbed bbc3 (Bcl-2-binding component 3) was also isolated by a yeast two-hybrid approach using Bcl-2 as bait (Han et al., 2001). Like Noxa, PUMA/Bbc3 delocalizes to the mitochondria, interacts with Bcl-2 through its BH3 domain, and induces cytochrome c release. Differential display has also been applied to reveal the expression of DP5/HRK (harakiri) in neurons upon nerve growth factor withdrawal (Imaizumi et al., 1997). Transcriptional induction of DP5/HRK is associated with apoptotic cell death in neuronal development and upon exposure of cultured neurons to β amyloid protein (Imaizumi et al., 1997, 1999).

Although transcription analysis was often not the starting point for cloning new cell death genes, transcriptional regulation of these genes can be crucial in the balance between live and death. The *C. elegans egl-1* (egg-laying defective 1) gene, which encodes a proapoptotic BH3-only protein, is transcriptionally repressed in the hermaphrodite specific neurons (HSN). These neurons are required for egg laying and are absent in male worms because male nematodes lack *egl-1* repression (Conradt and Horvitz, 1999). In fact, Egl-1 turned out to be a general cell death activator in *C. elegans*. Direct Egl-1–Ced-9 interaction overcomes the antiapoptotic effect of the Bcl-2-like protein CED-9 (cell death defective 9) (Conradt and Horvitz, 1998) and induces the release of WAH-1, the ortholog of AIF (apoptosis-inducing factor), an apoptogenic mitochondrial factor (Wang et al., 2002). Alternative splicing can provide an additional level of transcriptional regulation in cell

death. Pre-mRNA of Bim (Bcl-2-interacting mediator of cell death), which is inducible, gives rise to three splice variants, $Bim_{EL}$ (196 aas), $Bim_L$ (140 aas), and $Bim_S$ (110 aas), the latter displaying the highest cytotoxicity (O'Connor et al., 1998).

The use of microarray technology to analyze mRNA expression has uncovered a molecular mechanism induced in cardiac hypertrophy that can trigger cardiomyocyte cell death. Hypertrophy of the heart can be caused by a compensatory mechanism that allows the heart to cope with an increased labor demand as a result of hemodynamic stress, for example, imposed by artery obstruction. However, this condition predisposes to heart failure, which is associated with cardiomyocyte cell death. Overexpression of Gαq, the alpha component of the heterotrimeric Gq protein, induces cardiac hypertrophy. Yussman et al. compared the mRNA expression levels of normal and Gαq cardiac-specific overexpressing mice using microarrays (Yussman et al., 2002). This approach identified Nix (NIP3-like protein X), also named (B)NIP3L [(B)cl-2/adenovirus E1B 19 kD-interacting protein 3 Like], as one of the few cell-death-associated genes to be transcriptionally upregulated in this mouse model as well as in hypertensive cardiac hypertrophy patients. Moreover, it was demonstrated that transgenic, heart-specific overexpression of Nix causes cardiomyopathy. The induction and contribution of the BH3-only protein Nix/(B)NIP3L in cardiac hypertrophy and associated cardiomyocyte cell death are remarkable given that pro-apoptotic Bcl-2 family members are barely expressed in the developed heart (Cook et al., 1999). Cardiomyocyte loss in heart failure is not always associated with apoptotic cell death. Also autophagic and necrotic cell death phenotypes have been documented (Martinet et al., 2002; Ohno et al., 1998). In this respect, it is perhaps not surprising that (B)NIP3, a Nix homolog, has been shown to induce a necrotic type of cell death characterized by early plasma membrane permeability, mitochondrial damage, extensive cytoplasmic vacuolation, and mitochondrial autophagy (Vande Velde et al., 2000). Nix and (B)NIP3 share the ability to induce cell death that is not readily overcome by Bcl-2 and is not dependent on the BH3 domain. Unlike most other BH3-only proteins, they both form stable homodimers (Chen et al., 1999). Upregulation of Nix and (B)NIP3 at the transcript and protein level has also been documented in tumors and cardiomyocytes under hypoxic conditions (Guo et al., 2001; Kubasiak et al., 2002; Sowter et al., 2001). Tissue hypoxia can be the result of atherosclerotic plaque formation, which restricts blood flow and can cause the induction of a hypoxic condition in the tissue downstream from the affected region of the artery. Recently, it has been shown that hypoxia in combination with acidosis, as a result of increased glycolysis in hypoxia, leads to BNIP3-mediated caspase-independent cardiomyocyte death (Kubasiak et al., 2002). These findings illustrate the importance of Nix and BNIP3 expression regulation in a pathological condition, making these molecules interesting prognostic and therapeutic targets.

Clearly, transcriptional analysis has contributed substantially to unraveling cell death responses at the molecular level. Nevertheless, microarray data, and quantitative transcription data in general, should be scrutinized carefully as mRNA levels do not always reflect the relative amount of the encoded protein. In fact, both values may sometimes be opposites. An example of the latter is the transcriptional upregulation of the creatine kinase M chain during heart failure, as detected by microarray data, whereas the protein was actually downregulated, as deduced from two-dimensional gel electrophoresis (2-DE) data (Jiang et al., 2001). Another example where transcript and protein levels are not in accord is the downregulation of Rad51 protein expression in lung epithelial cells treated with TGFβ1. In an effort to identify new targets of this pleiotropic cytokine, Kanamoto et al. performed 2-DE analysis and showed that Rad51, an essential component of DNA double-strand break repair, is downregulated by TGFβ1 (Kanamoto et al., 2002). However, Rad51 mRNA levels were induced by TGFβ1. Proteasomal degradation of Rad51 turned out to be responsible for this discrepancy.

These examples illustrate the fact that it is imperative to consider translational and posttranslational control mechanisms when interpreting quantitative mRNA profiling data in order to draw conclusions based on gene product levels. Recently, it became clear that a number of apoptotic proteins such as XIAP and Apaf-1 contain an internal ribosomal entry site (IRES) to control their level of expression during cellular stress conditions (Holcik et al., 2000). An IRES is a structural element, usually located near the 5′ end of a mRNA, that allows its translation independent of a 5′ Cap. Cap-dependent translation is the normal mode of translation for most transcripts. However, when Cap-dependent translation is compromised, as, for example, can occur during stress conditions such as heat shock, hypoxia, or viral infection, IRES-dependent translation usually continues or is induced (Vagner, Galy and Pyronnet, 2001). Therefore, the use of the translated mRNA population, namely, the polysomal fraction, rather than the total mRNA pool for the generation of expression profile data could improve the accuracy of the information obtained (Pradet-Balade et al., 2001).

## Proteomics

Cellular functions are executed by proteins. So, in order to grasp how a cell responds to its environment and carries out its "housekeeping" biochemical functions, which in the context of development and homeostasis of multicellular organisms include programmed cell death, we need to understand the behavior and role of proteins in a global context. Proteomics, or proteome analysis, encompasses the methods that allow qualitative and quantitative protein analysis (Blackstock and Weir, 1999; Lee, 2001). In general, "classical" proteomic studies include (1) the isolation of the protein(s) of interest,

(2) separation of the isolated sample into its individual components, (3) visualization and quantification of the proteins, and (4) identification of the (selected) proteins (Figeys et al., 2001; Mann et al., 2001; Rappsilber and Mann, 2002). In the following sections, a brief overview of the available modern protein identification techniques and some examples of proteomics' contributions to cell death research will be presented.

## Protein Identification Techniques

Signal transduction pathways are traditionally studied using biochemical, genetic, and functional approaches. Identification of unknown signaling components using classical techniques, such as Edman degradation, requires large amounts of pure protein material. Mass spectrometry (MS) has revolutionized protein identification because it quickly measures the mass of biomolecular ions with extreme accuracy and sensitivity. Modern MS techniques are routinely used for protein identification and characterization, e.g., in classical biochemical studies, which in general allow the identification of a single isolated protein. On the other hand, large-scale proteome studies yield a global, holistic view of the protein content of a cell or an organelle under certain conditions and allow speculation about the connections and interactions among these components.

State-of-the-art Protein Identification Methods. The first step in proteomics is the separation of a protein mixture, generally by one- or two-dimensional polyacrylamide gel electrophoresis (1-DE or 2-DE), and the visualization of the components by staining techniques, the classic approach being the use of Coomassie brilliant blue or metallic silver. Coomassie staining is one of the least sensitive methods, requiring 10 to 100 ng of material, whereas silver staining is one of the most sensitive nonradioactive methods, visualizing proteins in the low nanogram range (<10 ng). Selected protein bands, for example, those for which the staining intensity and/or gel position changes between two cell states, are excised out of the gel and in gel digested, generally using a highly specific protease such as trypsin. The obtained peptide mixture is analyzed by MS and the obtained peptide mass fingerprint, which is unique for the investigated protein, can be used to identify the protein (reviewed by Cottrell, 1994). MS analysis, however, requires charged, gaseous molecules for analysis, a prerequisite, in the past, not easily matching large, polar biomolecules. Two ionization techniques, matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI), made this possible, allowing detection of biomolecules with more than reasonable throughput. Both MALDI and ES ionization techniques were developed in the late 1980s (Karas and Hillenkamp, 1988) and have been optimized since then (reviewed in Figeys et al., 2001; Mann et al., 2001). A third step in the identification of the proteins is the use of software algo-

rithms that link experimental MS data to protein and DNA sequence entries stored in databases. If, for instance, the excised protein band contained multiple proteins, then a peptide mass map may not lead to an unambiguous identification. In this case, individual peptides are selected and fragmented in the mass spectrometer, commonly by collision with an inert gas, such as argon or helium. The obtained peptide fragmentation spectrum contains valuable information about the sequence of the selected peptide and is used by software algorithms to link it to a peptide sequence stored in a database (Rappsilber and Mann, 2002).

*MALDI-MS.* MALDI is a technique in which the sample peptides or proteins are cocrystallized on a target plate with matrix compounds. Matrix molecules are usually small organic molecules that absorb the laser light used to irradiate the crystals in the ion source region of the mass spectrometer. The precise nature of this ionization process is still elusive, but generally leads to the formation of singly charged ions, either by protonation or deprotonation of amino acid side chains. MALDI has been predominantly coupled to time-of-flight (TOF) mass analyzers, which measure the time needed for peptide ions to reach a detector placed at the end of their flight path. Following electroextraction out of the ion source region, smaller peptide ions obtain higher velocities than bigger ones, and this is the basis of ion separation in the TOF region according to their mass/charge (m/z) ratio. When the mass spectrometer is calibrated using standard peptides of known mass, the TOF for any given ion can be converted into a highly accurate mass, resulting in a spectrum containing peptide ion intensities (*y*-axis) and peptide ion masses (*x*-axis). This set of empirical peptide masses is called a "peptide mass fingerprint"; it is subsequently compared to theoretical peptide mass maps generated by in silico digestion of (selected) sequences stored in a database (Cottrell, 1994).

This MALDI-MS mass fingerprinting method may however fail to identify the protein of interest, especially when working with complex protein mixtures, for example, when proteins are poorly resolved and the excised protein band contains multiple proteins (generally observed when analyzing one-dimensional PAGE separated proteins). Many MALDI-peptide ions fragment at their peptide bonds in the TOF region of the mass spectrometer, a phenomenon known as postsource decay (PSD) (Kaufmann et al., 1993). Upon fragmentation of the peptide backbone, two types of fragment ions are generally observed. Those containing the peptide's amino terminus are called $b_n$ ions and those containing the carboxyl terminus are called $y_n$ ions. Since the mass difference between two consecutive peptide fragment ions of the same type (e.g., between the $y_n$ and the $y_{n+1}$ ion) depends only on the nature of the amino acid side chain, PSD spectra can be used to sequence at least a part of the analyzed peptide (commonly called a peptide sequence tag). Nowadays, these spectra are routinely used to automatically identify

the original protein using bioinformatic tools without the need of any user interpretation (reviewed by Gevaert et al., 2001; van Loo et al., 2002a).

*ESI-MS.* In ESI, a liquid containing the sample is sprayed at a low flow rate through a needle with a small internal diameter, placed in front of the orifice of the mass spectrometer. Through the use of a high electrical field, the generated droplets travel to the inlet of the mass spectrometer, and with the aid of raised temperature and/or an inert drying gas, the droplet solvent rapidly evaporates. Eventually, gaseous, multiple-charged sample ions are generated (Fenn et al., 1989). With nano-electrospray, small sample volumes (1–2 μL) can be analyzed at very low flow rates (a few tens of nL/min), allowing highly in-depth analysis of complex peptide mixtures (Wilm et al., 1996). ESI mass spectrometers are routinely coupled in-line with a reverse-phase (RP) liquid chromatographic system, allowing the separation of complex peptide mixtures prior to mass spectrometric analysis. In most cases, ESI-based MS techniques are the methods of choice for peptide fragmentation studies (called tandem mass spectrometry or MS/MS) and the obtained information is used to identify the investigated protein(s). In such a setup, a particular peptide ion is first physically selected out of a mixture and then fragmented by collision to inert gas molecules, so-called collision-induced dissociation or CID. Since the obtained CID spectra are highly specific for the selected peptides, they can be finally linked to sequence entries stored in databases using commercially available database-searching tools [e.g., MASCOT (Perkins et al., 1999) and SEQUEST (MacCoss et al., 2002)] without any manual interpretation. Due to the nature of the investigated peptides and CID-fragmentation characteristics, in many cases, easily interpretable CID spectra are obtained. This can be used for de novo sequence analysis and even for the generation of degenerated oligonucleotide primers that can be finally used to clone the corresponding gene (Wilm et al., 1996). Furthermore, these types of mass spectrometric analyses are more and more routinely used for studying important amino acid modifications such as phosphorylation (reviewed by McLachlin and Chait, 2001) and glycosylation (reviewed by Dell and Morris, 2001).

*Gel-free Proteomics.* Over the last couple of years, novel peptide-centric mass-spectrometry-based approaches have been described that allow differential monitoring of protein expression in two different samples (e.g., Gygi et al., 1999). These approaches do not use polyacrylamide gel electrophoresis to separate the protein mixture. Instead, the protein sample is digested in solution, and either as many peptides as possible are analyzed (Washburn et al., 2001) or a specific set of representative peptides is isolated prior to analysis (Gygi et al., 1999; Gevaert et al., 2002). The main advantage of these gel-free techniques lies in the fact that most of the shortcomings of gel-based proteomics are no longer encountered. In gel-free proteome

studies, low abundant proteins next to highly hydrophobic proteins, which are classes of proteins that are very difficult to analyze by 2DE, are more frequently identified. Ultimately, this implies that a significantly larger number of the expressed proteome is covered (Gevaert et al., 2002), which leads to an increased understanding of the dynamics of a given proteome.

## Proteomics in Cell Death Research

### Detection of Protein Complexes.

Proteins usually do not operate alone but rather interact with other proteins to form transient or stable, small or large complexes, also referred to as "molecular machines" (Alberts, 1998). Sometimes, these protein assemblies are large enough to deduce their three-dimensional structure by cryo-electron microscopy, a technique which, for example, revealed the wheellike arrangement with 7-fold symmetry of the apoptosome (Acehan et al., 2002). Multiprotein complexes constitute the hardware of biochemical and signal transduction pathways. Therefore, a comprehensive view of the cell's protein–protein interaction map can lead to better insight into signaling responses at the molecular level and will help define novel drug targets to treat diseases. Yeast two-hybrid (Y2H), affinity capture, and gel filtration techniques are among the most widely and successfully applied techniques to study interactions and analyze multiprotein assemblies.

*Yeast Two-hybrid System.* Y2H is a genetic method for screening protein–protein interaction that in its original, still often used, setting is based on the reconstitution of a functional GAL4 transcription factor through the binding of two hybrid proteins. One contains the GAL4 DNA-binding domain fused to a "bait" protein; the other comprises the GAL4 activation domain fused to a potential bait-binding partner (the "prey"), often provided as a cDNA library (Fields and Song, 1989). This method has led to the identification of a host of novel Bcl-2 family members that, by mutual interaction between pro- and antiapoptotic members, control the trigger that initiates the intrinsic cell death pathway (Boyd et al., 1994; Cory and Adams, 2002; Yang et al., 1995).

Elucidation of the extrinsic, namely, the death domain receptor-mediated pathway to cellular demise, also profited from the Y2H method. Fas (CD95, APO-1) is an apoptosis-inducing receptor belonging to the TNF receptor superfamily, which contains several death domain receptors (Beyaert et al., 2002; Nagata, 1999). Cell death signaling by Fas requires the presence of its intracellular death domain (DD). Using the Y2H approach, two groups have isolated the cytoplasmic adaptor molecule FADD/MORT (Fas-associated death domain/mediator of receptor-induced toxicity). FADD homotypically associates with the DD of the ligated Fas/CD95 receptor and transduces the death stimulus by recruiting FLICE, now officially called

caspase-8 (Boldin et al., 1995; Chinnaiyan et al., 1995). Caspase-8, also named MACH (MORT1-associated CED-3 homolog), in turn was cloned by David Wallach's group using FADD/MORT as bait in Y2H screening (Boldin et al., 1996). Caspase-8 binds FADD/MORT through a DED–DED interaction motif, which resembles the DD, CARD, and PYRIN domains consisting of six to seven α helices with different topological orientations (Weber and Vincenz, 2001).

*Affinity-based Purification of Complexes.* Affinity capturing of a protein along with its possible associates is a direct biochemical approach to trace possible interaction partners. This method entails the immobilization of the protein of interest to allow its isolation from complex mixtures such as cell lysates. Immobilization is usually accomplished by an antibody directed to the bait protein or to its epitope-tagged counterpart. Other methods to enrich a protein of interest together with interacting molecules include its genetic fusion with heterologous domains such as gluthatione-S-transferase (GST), maltose-binding protein, or even a tandem arrangement of affinity modules, which allow specific adsorption to their respective immobilized ligand (Rigaut et al., 1999). A prerequisite for the affinity-capturing technique is that the protein bait maintains its native conformation and that the interactions be strong enough to withstand lysis and washing procedures during isolation of a particular complex. In addition, in order to allow MS-based identification of captured endogenous binding partner(s), a large amount of starting material is required. Despite these considerations, affinity-capture experiments have helped to clarify the Fas signaling pathway by studying the endogenous molecules that are recruited to the activated Fas receptor.

Activation of the Fas receptor by cross-linking, either with its natural ligand, FasL, or with an agonistic anti-Fas antibody, induces apoptosis in Fas-sensitive cells (Suda et al., 1993; Trauth et al., 1989; Yonehara et al., 1989). Fas signaling requires oligomerization into SDS-stable high molecular weight microaggregates that partition in membrane rafts (Dhein et al., 1992; Hueber et al., 2002; Kischkel et al., 1995). In an elegant approach advanced by Peter Krammer and coworkers, these proteins that physically associate in vivo with the activated receptor, and which they called cytotoxicity-dependent APO-1-associated proteins (CAP), were isolated and identified. CAP1 and CAP2 were identified as FADD. CAP-1 and -2 are both phosphorylated forms of FADD, as was shown by antibodies directed against the DD of FADD (Kischkel et al., 1995). Not long there after, procaspase-8 (CAP4) and the prodomain of caspase-8 (CAP3) were identified using MALDI mass spectrometry and nano-electrospray tandem mass spectrometry (nano-ES MS/MS) (Muzio et al., 1996). The latter method allows sequencing of femtomole quantities of proteins directly isolated from silver-stained gels (Wilm et al., 1996) (see above). Two other CAP proteins, CAP5 and CAP6, were

detected by 2-DE after prolonged Fas stimulation and identified as different forms of the caspase-8 prodomain using antibodies directed against different parts of caspase-8. The latter probably result from proteolysis at two different sites (Medema et al., 1997). This complex of intracellular signaling proteins that are recruited to the receptor upon activation was named the death-inducing signaling complex (DISC) (Kischkel et al., 1995). A similar approach, using recombinant soluble Flag-tagged TRAIL, was employed to purify affinity and analyze the death domain receptor TRAIL receptor-2, also called DR5, signaling complex (Bodmer et al., 2000). Like the Fas receptor, TRAIL-R2 activation induces the formation of a DISC that requires FADD and caspase-8 recruitment for its cytotoxic activity.

Flag-tagged or tandem affinity-tagged (TAP)-XIAP as well as GST-IAP-BIR3 fusions have been used by a number of groups to probe mammalian cell extracts for IAP-binding partners. Subsequent separation of the binding proteins by 1- or 2-DE followed by mass spectrometry has led to the identification of Smac/DIABLO and Omi/Htra2. Smac/DIABLO and the serine protease Omi/Htra2 were also identified by different approaches (see below); they turned out to be cell death agonists released from mitochondria that act, at least in part, as IAP antagonists (Hegde et al., 2002; Martins et al., 2002; Suzuki et al., 2001; van Loo et al., 2002b, c; Verhagen et al., 2000, 2002).

*Gel Filtration–based Purification of Complexes.* The mere size of multiprotein complexes permits their isolation by gel filtration in order to analyze their constituents and activity. This method has allowed the demonstration and functional characterization of the apoptosome. This is a high molecular weight caspase-activating complex that is formed by the scaffolding protein Apaf-1 (apoptosis protease-activating factor 1), cytochrome c, dATP, and cytosolic procaspase-9 (Cain et al., 1999, 2000; Saleh et al., 1999; Zou et al., 1999). In vitro activation of cell lysates with dATP induces the formation of two forms of the apoptosome: one of approximately 1.4 MDa and a second form of approximately 700 kDa (Cain et al., 2000). The latter form is the most prominent in apoptotic cells and is more active in processing procaspase-9 and -3 than the 1.4 MDa complex that seems to harbor Apaf-1 in an altered conformation (Bratton et al., 2001a). Caspase-3 and -7 are also associated with the apoptosome, but because these caspases are removed by washing, the apoptosome with a low salt (50 mM) concentration buffer, their association is not very strong (Cain et al., 2000). Remarkably, XIAP is also readily recruited to the apoptosome where it can prevent full caspase-9 (and -3) processing by binding to the amino terminus of the small subunit of caspase-9, which is generated upon the first autocatalytic cleavage at position D315 of human pro-caspase-9 in the apoptosome (Bratton et al., 2001b; Srinivasula et al., 2001). Caspase-3-dependent cleavage of pro-caspase-9 at position 315 generates a caspase-9 form that is not able to bind XIAP (Srinivasula et al., 2001). This subtle inhibitory mechanism may have

evolved to prevent inadvertent caspase-9 activation. However, competition for XIAP-binding by the mitochondrial factors Smac/DIABLO and Omi/Htra2 (reviewed by van Loo et al., 2002b) can overcome this inhibition.

In analogy with the apoptosome, the recent discovery of the "inflammasome," a multiprotein complex that activates inflammatory caspases to generate active IL-1β, suggests that the recruitment of a large prodomain containing caspases into high molecular weight assemblies is a prevailing prerequisite for their activation (Martinon et al., 2002). The formation of the inflammasome, which was demonstrated by gel filtration analysis of cell lysates, relies on the presence of the apoptosis-associated specklike protein containing a CARD (ASC), also named the Pyrin- and CARD-containing protein (PYCARD) (Martinon et al., 2002).

Role of Mitochondria in Cell Death. It is now obvious and well accepted that mitochondria integrate apoptogenic signals from various origins by initiating the intrinsic cell death pathway. The concept that different organelles may act as sensors for stress signals which can eventually converge on the release of mitochondrial factors has been extensively reviewed (Ferri and Kroemer, 2001). Important aspects of these sensing functions are phosphorylation and dephosphorylation status of pro-apoptotic Bcl-2 family member proteins (reviewed by Cory and Adams, 2002), proteolysis of target proteins, and subcellular relocation of proteins. It is obvious that lysosomal proteases, mitochondrial factors, BH3-only proteins, DNAses, and proteases gain their cell death function only when targeted in the proper complex or organelle, or released in the cytosol. The relocation of apoptogenic factors is an important issue in cell death signaling. One approach to studying this is the in vitro reconstitution of a pathway by combining subcellular fractions such as cytosol, mitochondria, and nuclei. In this way, Xiaodong Wang using large-scale biochemical purification steps has identified mitochondrial factors that once added to cytosol were able to activate caspases and to induce internucleosomal DNA fragmentation on isolated nuclei (Li, Luo and Wang, 1997; Zou et al., 1997). This experimental approach was a milestone in unraveling the molecular mechanisms of the intrinsic cell death pathway leading to a cytochrome-c-dependent generation of the apoptosome complex and the activation of a caspase cascade. In a similar approach, his group identified a cytosolic factor that was proteolytically activated by recombinant caspase-8 to cause cytochrome c release from isolated mitochondria, namely, Bid, a BH3-only member of the Bcl-2 family of proteins (Luo et al., 1998).

Besides cytochrome c, other apoptogenic proteins, such as apoptosis-inducing factor (AIF) and Smac/DIABLO, are released from the intermembrane space of mitochondria to the cytosol of cells that have received an apoptotic stimulus (Du et al., 2000; Susin et al., 1999; Verhagen et al., 2000). To identify other proteins released from mitochondria in a tBid-dependent

way, we used an in vitro reconstitution system in which isolated mouse liver mitochondria were treated with purified recombinant tBid. The proteins released from the isolated mitochondria were identified by MALDI-PSD MS; see above (van Loo et al., 2002a). This approach led to the identification of cytochrome c, Smac/DIABLO, adenylate kinase 2 (AK2), and some new proteins, such as endonuclease G, a mitochondrial nuclease involved in caspase-independent DNA degradation (Li et al., 2001; van Loo et al., 2001), and the serine protease Omi/HtrA2. The latter, just like Smac/DIABLO, can inhibit IAP proteins, but also has cell-death-inducing activity dependent on its catalytic serine protease property (Hegde et al., 2002; Martins et al., 2002; Suzuki et al., 2001; van Loo et al., 2002c; Verhagen et al., 2002).

It can be expected that similar experimental approaches using subcellular fractions and recombinant proteins implicated in cell death will lead to the identification of new molecular mechanisms that affect the turnover of organelles such as lysosomes, autophagosomes, mitochondria, peroxisomes, nuclei, and endoplasmic reticulum and explain how these organelles interact with each other in the process of cell death. The in vitro reconstitution assays combine old, somewhat forgotten technologies of organelle preparation by density gradient ultracentrifugation with powerful and sensitive methods in MS.

Proteomics of the Dying Cell. In an attempt to obtain a holistic view of the protein changes occurring during cell death, several groups have performed high throughput proteome analyses and cataloged the observed protein alterations in the whole cell associated with the progression of the cell death program. In the following section we will give one example of such an approach. Gerner and colleagues focused on the cytosolic protein fraction and attempted to unravel the underlying mechanisms of the observed protein alterations occurring in the dying cell. To this end, proteome alterations were correlated with de novo protein synthesis, protein translocation, or posttranslational modification (Gerner et al., 2000). By comparing 2-DE patterns of control cytosol with cytosol from Jurkat cells treated for 5 hours with an agonistic anti-Fas antibody, 19 silver-stained spots were found to decrease and 38 spots to increase after anti-Fas treatment, whereas the majority of proteins, around 1,000 spots, seemed unaffected. The observation of caspase-3 processing served as an internal control. Differential spots in control and apoptotic conditions were identified by mass spectrometry. By comparative 2-DE analysis of protein from a $^{35}$S-Met/Cys labeling setup started 3 hours after anti-Fas treatment, it was shown that hsp27 and hsp70B, among others, appeared because of de novo synthesis. The latter experiment seems somewhat odd because several groups have reported on the rapid drop in translational activity in most apoptotic cells, including Jurkat T cells (Clemens et al., 2000; Saelens et al., 2001). This discrepancy may be due to de novo protein synthesis association with a stress response

in the nondying part of the population. $^{32}P$ labeling demonstrated changes in the phosphoproteome of the dying cells. Also a number of proteins were identified that relocate from the cytosol to the nucleus or vice versa. A fourth type of modification was the proteolytic fragmentation of certain proteins in apoptotic conditions. This became apparent with the disappearance of spots under apoptotic conditions associated with the appearance of spots of fragments of the same proteins at other positions in the 2-DE. In addition, the proteome alteration profile of necrotically dying Jurkat cells was analyzed. For these studies, Jurkat cells were treated with oligomycin, an inhibitor of the ATP synthetase (complex V) of the oxidative phosphorylation at the inner membrane of mitochondria. Overall, very few of the identified proteins displaying alterations in anti-Fas-killing were also altered in the necrotic setting, suggesting that most of the proteomic alterations observed in the anti-Fas-treated cells were related to the execution of the apoptotic pathway and not the result of mere cellular demise. This high throughput proteome analysis of a dying cell population essentially has provided an inventory of protein alterations occurring during cell death, with some preliminary information on the cause of these alterations (de novo synthesis, proteolysis, phosphorylation, relocation). However, a global functional understanding of these proteome alterations will need further investigation that sheds light on the function of sets of proteins under normal as well as pathophysiological circumstances (Huber, 2003; Rappsilber and Mann, 2002).

Degradomics: Functional Profiling of Proteases. The function of a protein in its cellular environment is determined in many ways, including by its expression level in space and time, its interaction with other proteins and its posttranslational modification, such as phosphorylation/dephosphorylation and proteolysis. These control mechanisms are dynamic and allow the cell to swiftly respond to its environment. Therefore, the correlation between the mere presence or expression level of a protein, for example, a protease or a kinase, and its activation state or participation in cellular function, is mostly not obvious. Therefore, techniques are being developed that allow profiling of a protein's posttranslational modifications and activity (Kuster et al., 2001; Mann et al., 2002). Because of the important role of proteases in cell death, we will draw attention to some recent progress in the development of degradomics, the application of genomic and proteomic approaches to identify and characterize proteases, their activity, substrates and inhibitors to unravel the role of proteases in vivo (Lopez-Otin and Overall, 2002).

Using a colorimetric DEVD-peptide cleavage assay to monitor caspase-3 activity in HeLa S-100 extracts, the group of Xiaodong Wang identified α-(trichloromethyl)-4-pyridineethanol (PETCM) from a 184,000 compound library screening as a potent activator of caspase-3 in the cytosol. PETCM activated caspase-3 in these lysates by inducing the formation of the apop-

tosome complex. By further fractionation of the HeLa cell lysate, the tumor suppressor putative HLA-DR-associated proteins (PHAP) and the oncoprotein prothymosin-α (ProtT) were discovered as a stimulator of apoptosome activity and an inhibitor of apoptosome formation, respectively. PETCM was shown to relieve ProtP-mediated inhibition of apoptosome formation, explaining its selection in the drug screening. This relief by PETCM could not be reproduced in an in vitro reconstituted system containing purified Apaf-1, procaspase-9 cytochrome c, PHAP, and ProT, suggesting that an additional, so far unknown, cytosolic factor is required. These results illustrate how the use of a defined protease activity readout (apoptosome activity) in a drug screening can lead to the dissection of a novel regulatory pathway in cell death.

To visualize caspase activity inside a cell in a facile, noninvasive way, fluorescence resonance energy transfer (FRET) technology has been applied by linking two fluorescent proteins, for example, green (GFP) and blue fluorescent protein (BFP), with a short-peptide sequence containing the caspase cleavage site (Xu et al., 1998). Cleavage of this linkage by a caspase eliminates the FRET effect and allows visualizing caspase activity by monitoring the shift in the fluorescence spectrum that occurs upon cleavage. This technique has also been used in drug screening to identify small-molecule inducers of apoptosis (Jones et al., 2000).

Because the catalytic mechanism of caspases at some stage involves the formation of a covalently bound acyl-enzyme intermediate (reviewed by Lamkanfi et al., 2003), compounds were developed that are based on the stabilization of this intermediate by using an irreversible inhibitor, for example, the pan-caspase inhibitor z-VAD-fluoromethylketone. The latter functional group binds covalently with the catalytic cysteine of an active caspase. Linkage of the inhibitor to an immobilizable ligand such as biotin permits the selective pulldown of the pool of active caspases. Based on this principle, an elegant profiling approach to caspase activity was recently reported by Winsssinger et al. (2002). The authors probed a cell lysate using a small-molecule inhibitor covalently linked to a unique peptide nucleic acid (PNA) (Fig. 1). The PNA moiety has a dual purpose. First, its sequence allows the identification of the attached small molecule, which is important if candidate inhibitors from a library of small molecules are tested as a pool. Second, it permits immobilization of the inhibitor, after interaction with its target protease, to a specific location in an oligonucleotide microarray by hybridization to a complementary sequence. Since the PNA part is also fluorescently labeled, it allows location and quantification of the signal in the microarray setting. In order to profile enzymatic activities in a complex sample, the small-molecule moiety was designed to serve as a mechanism-base inhibitor that can form a covalent association with the active enzyme(s) of interest. As proof of principle of their technique, the authors made use of a peptide-acrylate (the small-molecule moiety) that covalently and irreversibly

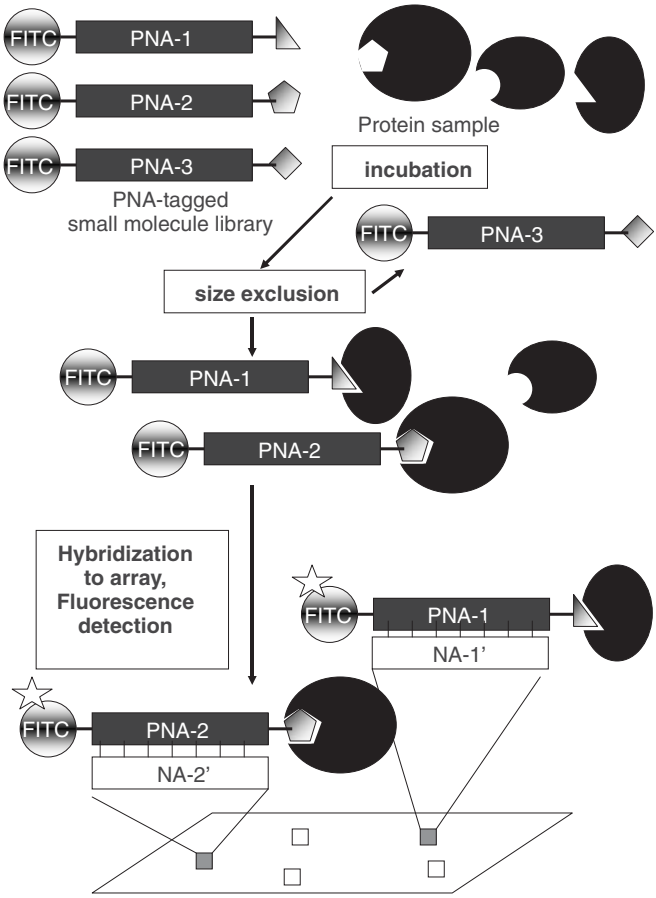


FIGURE 1. Overview of peptide nucleic acid (PNA)-small-molecule-based affinity screening. A library of small molecules (top left) consists of a pool of unique small molecules with potential affinity for a target in a complex mixture, for example, a cell lysate (protein sample, top right). Each individual small molecule is coupled to a unique PNA tag. The nucleotide sequence (PNA-1, -2, -3) of this tag encodes the chemical nature of the small molecule, is complementary to a dedicated oligonucleotide sequence (NA-1′, NA-2′, bottom part of figure) present in an array, and is labeled with an FITC fluorogen to allow quantitative detection. After incubation of the small-molecule library and the protein sample to allow covalent interaction, free PNA-small molecules are removed by size exclusion and the high molecular weight fraction is applied to an oligonucleotide array. This allows the hybridization between complementary NA strands and quantification of the immobilized protein-small-molecule complex.

modifies nucleophylic thiols such as the catalytic site of active cysteine proteases. The selectivity for a particular cysteine protease, in this case caspase-3, was governed by using Asp-Glu-Val-acrylateAsp as the peptide-acrylate. This peptide-acrylate-PNA was incubated with apoptotic cell lysate from Jurkat T cells followed by removal of free peptide-acrylate-PNA molecules by a size exclusion step. Hybridization of the resulting fraction on a microarray yielded a fluorescent signal at the expected location and with an intensity that correlated with the amount of active caspase-3 present in the sample. MS/MS data analysis of the protein immobilized by the peptide-acrylate probe identified human caspase-3 as its specific target.

## Conclusions and Future Perspectives

This chapter has focused on the available technologies that have assisted and will continue to assist in identifying and unraveling the role of proteins in death signaling pathways. These technologies cover a range of experimental methods going from classical biochemical approaches that have led, for example, to the identification of caspase-1 (Thornberry et al., 1992) to yeast two-hybrid to modern-day genomics, transcriptomics, and high throughput proteomics. The technology that allows the capture and identification of proteins from complex mixtures is evolving at a continuous pace. Two main challenges that need to be tackled in proteomics-based research are sample preparation and protein activity measurements. This includes the development of techniques that allow one to analyze the proteome from very small samples from a few cells and that allow the simultaneous analysis of a highly complex mixture of proteins. On the other hand, sensitive techniques that allow the measurement or labeling of active proteins will help to interpret the metabolic state of cells and subcellular compartments. Without an amplification method at hand, such as the polymerase chain reaction for nucleic acid research, it is difficult to envision an approach that will eventually permit cataloging the proteome of only a few cells, since otherwise only the most abundant proteins will be detected.

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CHAPTER

17

# CELL DEATH IN VIRAL INFECTIONS

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Viral infection is often accompanied by the death of host cells. Living as intracellular parasites, viruses have adapted to employ cellular replication, transcription, and translation machinery for their own benefits. The disruption of normal cellular functions or damage to cellular structures was once thought to explain how viruses forced host cells to die. It was only until the concept of programmed cell death (PCD) was proposed that cell death caused by viral infection was recognized as altruistic suicide through specialized cellular machinery. In multicellular organisms, the activation of cellular suicide program in response to virus infections is now considered to be an effective early defense strategy to prevent the spread of infection, reducing the virus burden for the later, more specific immune-mediated execution of infected cells. Therefore, this altruistic suicide of infected cells is a crucial component of the host defense system. The suicide program by which cells die upon infection is often called apoptosis, but is more broadly referred to as PCD because not all genetically programmed deaths exhibit morphological and biochemical characteristics of apoptosis.

Dying by PCD involves a cascade of events that is regulated by a wide variety of cellular factors which either inhibit or facilitate the program. The classical form of PCD, or apoptosis, is characterized by distinct morphology changes, including chromatin condensation, DNA fragmentation to

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nucleosome-sized pieces, membrane blebbing, cell shrinkage, and compartmentalization of the dead cells into membrane-enclosed vesicles or apoptotic bodies that are engulfed by neighboring cells. Cell death induced by some viruses exhibits these characteristic apoptotic morphologies. However, many viruses have devised methods to suppress the programmed death pathway of their host cells. In such situations where the infected cell ultimately dies, the morphology of the dying cell may be primarily that of necrosis rather than apoptosis. Also in some virus-infected tissue localities, the extent of apoptosis can be severe. The presence of apoptotic bodies bearing foreign viral antigens, or perhaps the eventual lysis of accumulated unengulfed apoptotic bodies may contribute to the intense inflammatory response observed in some virus infections. In these situations, the immune response is often more damaging than the virus. In this regard, virus-induced apoptosis can differ from developmental or homeostatic apoptosis, which is generally noninflammatory. However, a complicated relationship exists between inflammatory immune responses and the cell death pathway that has not yet been clarified. Virus-infected cells can also be eliminated by natural killer (NK) cells or cytotoxic T cells (CTLs) and these processes have been attributed to programmed cell death. These immune cells either secrete cytotoxic cytokines such as tumor necrosis factor (TNF) or deliver FasL (Fas ligand) to one or more death receptors on the cell surface, or release perforin or granzymes into targeted virus-infected cells based on antigen recognition (Tortorella et al., 2000).

The fact that many viruses are found to encode genes that inhibit the cellular apoptotic process strongly supports the concept that virus-activated cellular suicide programs have evolved as an effective antiviral strategy. E1B of adenovirus and P35 of baculovirus were the first identified viral antiapoptotic genes, and mutation of these genes in the viral genome causes severe impairment of progeny virus production apparently due to premature death of host cells (Clem et al., 1991; Clem and Miller, 1993; White, 2001; White et al., 1984). In addition to E1B-19K and P35, many other antiapoptotic genes have been found in the genomes of numerous viruses. Thus, apoptosis was proposed as a mechanism to prevent viruses from completing their replication cycles and producing progeny, thereby blocking the spread of infection. However, there may be other protective roles of apoptosis as it has also been observed that many animal viruses grow efficiently in cells undergoing apoptosis. For example, phagocytosis of apoptotic bodies may be an important route to present viral antigens and initiate an acquired immune response (Koyama et al., 2000).

In contrast, apoptosis is not always beneficial for the host. Certainly, apoptosis is envisaged as the mechanism of choice to rid the host of virus-infected cells, and failure to do so often results in viral persistence. However, continuing to harbor a virus for the life of the host may be a more desirable outcome if the price of deleting virus-infected cells is the elimination of postmitotic neurons of the central nervous system or other irreplaceable cell

population. As in the cases of the neuronotropic poliovirus or herpes simplex virus, these viruses induce neuronal cell death in motor neurons or temporal lobe neurons, respectively, and the extent of neuron loss determines the severity of disease or rate of fatality. Human immunodeficiency virus (HIV) challenges us with an even more complicated scenario. HIV encodes several genes (tat, nef, vpr, etc.) that have been reported to have both antiapoptotic and pro-apoptotic functions. Indeed, this virus likely employs both anti- and pro-apoptotic mechanisms that preserve the latently infected cell and facilitate transmission, but at the same time contributes to the relentless death of CD4+ lymphocytes characteristic of the later stages of disease and loss of immune competence in patients. Therefore, both inhibition and enhancement of apoptosis can contribute to viral pathogenesis. In the past few years, we have started to understand many aspects of programmed cell death regulation, yet the significance and net effect of apoptosis on the consequence of virus infection are a territory that is poorly understood.

Recent research into virus-infection-mediated cell death has identified new viral genes that modulate the host cellular apoptotic response. These investigations have not only expanded the possibility of developing new antiviral agents, but have also greatly deepened our understanding of the molecular mechanisms of cellular pathways. In this chapter, we first review the viral gene products that inhibit the initiation or execution phases of apoptosis at multiple levels in the pathway, followed by a discussion of the mechanisms involved in virus-induced cell death.

## Inhibition of Apoptosis by Viruses

Viruses interfere with the cell death pathway at almost every possible point (Roulston et al., 1999). The responsible viral proteins have been identified in many cases and have been grouped into several major categories according to where they act in the cellular apoptotic pathways (Table 1). Some examples are discussed in the following sections and their points of interface with cellular apoptosis pathways are summarized in Fig. 1.

### Inhibitors of Death-receptor-mediated Apoptosis

Death receptors are members of the tumor necrosis factor receptor (TNFR) superfamily, and have a cytoplasmic death domain (DD) required for relaying extrinsic death signals into the cell. Binding of specific ligands to these receptors induces trimerization or other conformational changes that translate to their cytoplasmic tails, leading to the formation of protein complexes referred to as the death-inducing signaling complex (DISC). Adaptor proteins are recruited to the DISC complex through DD interactions and activate a series of intercellular events that lead to diverse biological processes,

TABLE 1. Viral Inhibitors of Apoptosis

| Homologues/mechanisms | Virus | Viral Inhibitors |
|---|---|---|
| *Inhibitors of Death-receptor-mediated Apoptosis* | | |
| TNF receptor mimics | Cowpox virus | CrmB,C,D |
| TNF receptor mimics | Myxoma virus | M-T2 |
| Degradation of death receptors | Adenovirus | RID (E3-10.4/14.5K) |
| vFLIP | Pox virus MCV | MC159/MC160 |
| | γ-herpes viruses | |
| vFLIP | EHV-2 | E8 |
| vFLIP | HHV-8/KSHV | K13 |
| vFLIP | HVS | Orf 71 |
| Blocks ASK activity | HIV | Nef |
| *Bcl-2 Homologue* | EBV | BHRF1 |
| Inhibits pro-death cellular Bcl-2 family proteins and maintains mitochondrial integrity during cell death by an unknown biochemical mechanism | HHV-8/KSHV | KSBcl-2 |
| | HVS | Orf 16 |
| | ASFV | A179L |
| | Murine γ-68 | M11 |
| | Adenovirus | E1B 19K |
| | EBV | BARF |
| *Inhibitors That Act at Mitochondria* | | |
| Blocks permeability transition | Myxoma poxvirus | M11L |
| Binds ANT | CMV | vMIA |
| *Caspase and Granzyme B Inhibitors* | | |
| Serpin | Cowpox virus | CrmA |
| No homologue identified | Baculovirus | P35 |
| IAP | Baculovirus | Cp-IAP |
| IAP | Baculovirus | Op-IAP |
| Granzyme B inhibitor | Adenovirus | L4-100K |
| *Inhibitors of p53* | | |
| Inhibits activity; degradation | Adenovirus | E1B55K |
| Inhibits activity; degradation | Adenovirus | E4orf6 |
| Binds to and inactivates p53 | SV40 | Large TAg |
| Targets p53 for degradation | HPV | E6 |
| *Inhibitors Enhancing Survival Signals* | | |
| Activates Akt, phosphorylates BAD | Polyoma virus | Middle TAg |
| Activates PAK, phosphorylates BAD | HIV | Nef |
| Induces PI3K activation | HIV | Tat |
| Induces PI3K activation | Hepatitis B | HBx |

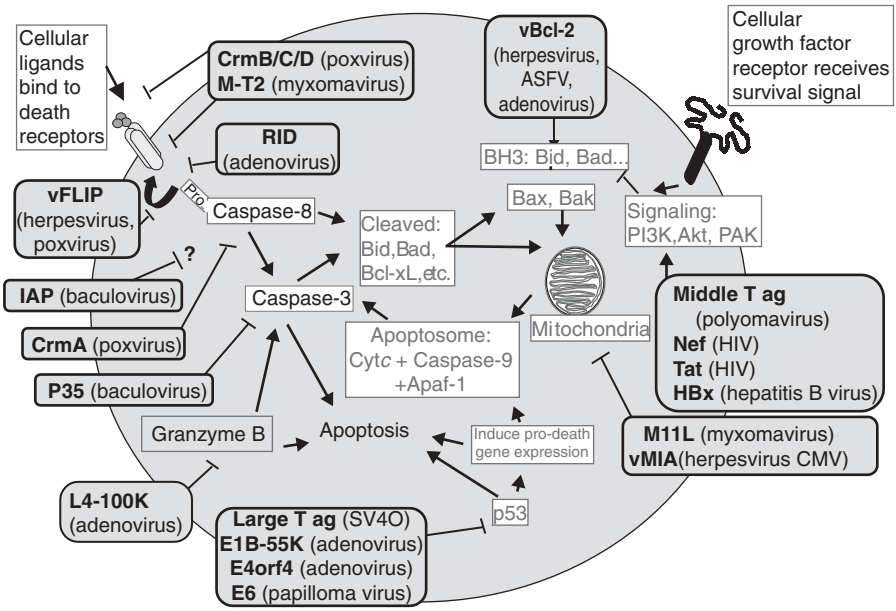


FIGURE 1. Viral proteins that inhibit programmed cell death. Viral proteins (bold) encoded by the indicated viruses (rounded boxes) interface with cellular factors (red). Arrows indicate direction of the pathway, blunt lines inhibitory actions.

including cell death, proliferation, inflammatory and stress responses (Ashkenazi and Dixit, 1998; Chen and Goeddel, 2002). One of these adapters, FADD (Fas-associated death-domain-containing protein), recruits and induces autoactivation of caspase-8 (FLICE), thereby initiating the apoptotic caspase cascade. As this extrinsic cell death pathway is extensively involved in antiviral immune responses, it is not surprising to see a variety of inhibitory plots invented by viruses.

Pox viruses devote approximately a quarter of their genome to genes that are not required for virus replication but are presumably critical for survival of the virus in the environment. Among these nonessential genes are mimics of TNFR that come in several forms, secreted, associated with the cell surface or intracellular. When the cytokine response modifiers CrmB, CrmC, and CrmD encoded by cowpox virus are secreted at different stages of infection, they can bind and neutralize TNF. Although the role of these proteins in regulating inflammatory responses of the host is well documented, their role in apoptosis inhibition in the infected host is less clear (Hu et al., 1994; Loparev et al., 1998; Smith et al., 1996). The M-T2 protein of myxoma virus, another pox virus, not only has a secreted form that blocks TNF signaling, but also an intracellular form that cannot bind to TNF

directly, yet still protects infected lymphocytes from apoptosis probably through abrogating distinct cellular responses (Nash et al., 1999; Xu et al., 2000).

Adenovirus adopts another strategy to desensitize cells to TNF-induced death. RID (receptor internalization and degradation) complex is composed of E3-10.4K and E3-14.5K that mediate rapid internalization and lysosomal degradation of cell surface Fas and another death receptor TRAIL-R1 (Tollefson et al., 1998, 2001). By removal of death receptors from the cell surface, the RID complex grants protection to infected cells from cytotoxic immune response.

In the DISC complex, adapter proteins such as FADD have a DD to bind the receptor and a related death effector domain (DED) to recruit caspase-8 through interactions with the DED domains present in the N-terminal pro-domain of caspase-8. Therefore, interference with these interactions serves to block the activation of caspases. Several γ-herpes viruses and MCV (molluscum contagiosum virus), a pox virus, are found to encode viral FLICE/capase-8 inhibitory proteins (vFLIPs). vFLIP proteins mimic the pro-domains of caspases in that they contain two DED domains and bind to FADD and/or caspase-8 and -10, inhibiting activation of initiator caspases after death receptor ligation (Thome et al., 1997). However, the details of their inhibitory mechanisms are still under investigation. The identification of vFLIPs instantly led to the identification of cellular FLIPs (cFLIPs), which have two forms; the short form contains two death effector domains and is structurally related to vFLIPs, whereas the long form, cFLIP(L), contains an additional caspaselike domain but without proteolytic activity. The cFLIP proteins are predominantly expressed in muscle, lymphoid tissue, and some tumor cells, strongly suggesting the critical role of cFLIPs as endogenous modulators of apoptosis (Irmler et al., 1997). Recent evidence suggests cFLIP and vFLIP are also involved in the regulation of NF-κB and c-Fos activation downstream of TNF receptors (Kataoka et al., 2000; Liu et al., 2002; Siegmund et al., 2001).

Many viruses trigger an upregulation of Fas and/or TNFR in infected cells. When these cells encounter TNF or other death signals, stress-activated protein kinase, also known as c-Jun NH(2)-terminal kinase (JNK), is activated in cells treated with TNF and can mediate both transcription-dependent (e.g., upregulation of FasL) and transcription-independent (e.g., mitochondrial cytochrome c release) apoptotic signaling (Davis, 2000). Apoptosis signal-regulating kinase 1 (ASK1) is a MAPKKK family member, and its activation by TRAF2 (TNF-receptor-associated protein 2) is important for TNF-mediated activation of JNK in signaling cell death (Ichijo et al., 1997; Nishitoh et al., 1998; Tobiume et al., 2001). HIV-1 does not encode a FLIP protein. Instead, it takes a different tack and utilizes a multifunctional protein, Nef, to bind and inhibit ASK1 activity, thereby blocking JNK activation and subsequent cell death (Geleziunas et al., 2001).

## Bcl-2 Homologues

Cellular Bcl-2 family proteins are key regulators of apoptosis. At the mitochondrial level, they can regulate permeability of the outer mitochondrial membrane, blocking or promoting the release of pro-death factors such as cytochrome c, SMAC/Diablo, AIF, EndoG, and HtrA/OMI from the intermembrane space. Yet the biochemical mechanisms by which Bcl-2 family members achieve these functions are still a mystery. Generally, Bcl-2 proteins are divided into three subgroups. Antiapoptotic family members include Bcl-2, Bcl-xL, Mcl-1, and Bcl-w, and often have the full complement of BH (Bcl-2 homology) domains. The multidomain pro-apoptotic family members such as Bax and Bak generally have three of the four BH domains. A subset of pro-apoptotic family members has only a short 12 to 15 amino acid motif that has recognizable sequence homology with the Bcl-2 family, and these proteins are referred to as BH3-only proteins. Although these classifications generally apply, the functions of Bcl-2 family proteins can be reversed. That is, Bax can be antiapoptotic (Lewis et al., 1999) and Bcl-2 can promote cell death (Cheng et al., 1997; Clem et al., 1998). Their abilities to regulate apoptosis are dependent on cell type and the particular death stimulus. However, until the biochemical functions of Bcl-2 family proteins are known, their reversible functions remain an enigma. The current working hypothesis is that the antiapoptotic proteins bind and inhibit BH3-only proteins, preventing them from activating the multidomain pro-apoptotic members Bax and Bak (Cheng et al., 2001). Upon receiving a death stimulus, Bax is translocated from the cytosol to mitochondria where it promotes the release of apoptogenic mitochondrial factors into cytosol. The mechanism by which Bax facilitates this release is still debated, but it has been suggested that it involves formation or induction of channels in the outer mitochondrial membrane. However, other possibilities remain and these issues are still far from clear (Adams and Cory, 2001; Martinou and Green, 2001).

Viral Bcl-2 homologues have been found in γ-herpes viruses, including human EBV and Kaposi's sarcoma-associated herpes virus (KSHV) and in the unrelated deoxyvirus African swine fever virus (ASFV). Even though the viral homologues found share only low amino acid sequence similarity with cellular Bcl-2 proteins (20%), the NMR structure of KSBcl-2 from KSHV is quite similar to that of Bcl-$x_L$ and Bax (Huang et al., 2002; Muchmore et al., 1996; Suzuki et al., 2000). In addition to herpes viruses, adenovirus also encodes a functional Bcl-2 homologue, E1B-19K. E1B-19K lacks significant overall sequence similarity to the Bcl-2 family, but it still remains possible that its 3-dimensional fold will be similar to that of other members of the Bcl-2 family when the structure is known.

Most viral Bcl-2 homologues contain only the BH1 and BH2 domains, but with poorly conserved or unrecognizable BH4 and BH3 domains. The N-terminal BH4 domain is required for the antiapoptotic function of cellu-

lar Bcl-2, but this domain is poorly preserved among cellular as well as viral Bcl-2 family members, perhaps suggesting novel functions or interactions. The BH3 domain is thought to be important for pro-apoptotic function; therefore, it is not surprising that viral Bcl-2 homologues have a poorly conserved BH3. However, the structure of KSBcl-2 reveals that alpha helices corresponding to the BH4 and BH3 domains are retained in viral Bcl-2. Like their cellular counterparts, the mechanisms by which viral Bcl-2 inhibits cell death are not yet clear.

Poor conservation of the BH3 sequence motif among viral Bcl-2 proteins correlates with the finding that viral Bcl-2 proteins are only capable of anti-apoptotic function and cannot be converted into pro-death factors in a manner similar to cellular Bcl-2 and Bcl-$x_L$ (Bellows et al., 2000). Unlike their cellular relatives, viral Bcl-2 proteins are resistant to proteolysis in a loop domain between BH4 and BH3. Cleavage by caspases or other proteases in this loop region converts Bcl-2 and Bcl-xL into killer proteins, and further enhances the killing activity of Bax, Bad, and Bid. Furthermore, deletion of N-terminus of viral Bcl-2 proteins to mimic caspase cleavage products generally fails to reveal any pro-death activity. These key differences between cellular and viral homologues provide one possible mechanism for viruses to escape cellular regulation. Another example of vBcl-2 deregulation is seen in KSHV, which encodes a viral cyclin homologue that directs cellular cyclin-dependent kinase (CDK) 6 to phosphorylate cellular Bcl-2, leading to its degradation. In contrast, KSBcl-2 is not a substrate of the viral cyclin-CDK6 complex, thereby escaping the degradation process (Ojala et al., 2000).

Despite the compelling evidence that viral Bcl-2 homologues inhibit apoptosis in vitro, the role of these viral Bcl-2 homologues in vivo remains unclear. Most γ-herpes viruses express their Bcl-2 homologues early in the lytic cycle, suggesting a role for these proteins in prolonging cell survival during virus replication, rather than a role during latency and subsequent tumor formation. However, the expression pattern of herpes virus Bcl-2 proteins remains unresolved, and verification that the murine γ-herpes virus 68 (γHV68) expresses its Bcl-2 homologue during latency as well as the lytic cycle fuels the earlier but unconvincing evidence that other herpes viruses may be capable of the same. Interestingly, recent studies using a mouse model to study γHV68 have provided valuable new information over the earlier cell culture models. In vitro studies of EBV mutants lacking vBcl-2 revealed no detectable change in virus replication and immortalization of primary human B cells (Marchini et al., 1991). Consistent with this, the vBcl-2 of the γHV68 virus was also not essential for acute replication, establishment of latency, or for virulence in vivo. More importantly, vBcl-2 was required for efficient reactivation from latent infection, and Bcl-2-defective viruses had markedly reduced pathological consequences in immunocompromised (IFN-$\gamma^{-/-}$) mice compared to wild-type virus. This study provides the first evidence that vBcl-2 contributes to both persistent replication and virulence during chronic infection (Gangappa et al., 2002). Even though each

γ-herpes virus is highly adapted to its specific host, the lessons learned from γHV68 may help unravel the complicated interaction of the human viruses EBV and KSHV where other factors also contribute to development of herpes-virus-associated tumors, including status of immune competency, and environmental and genetic factors.

Adenovirus E1B-19K blocks E1A-induced, p53-dependent or -independent apoptosis through its binding to Bak or activated Bax at the mitochondrial membrane. Thus, E1B-19K abrogates the potential pore-forming ability of Bak and Bax, and has been extensively reviewed elsewhere (White, 2001).

## Other Viral Cell Death Inhibitors Acting on Mitochondria

In addition to viral Bcl-2 homologues, there are other viral proteins that inhibit apoptosis at the level of mitochondria. Like Bcl-2 proteins, these viral factors also prevent loss of mitochondrial membrane potential and permeabilization of the outer membrane. For example, the myxoma pox virus protein M11L, which is required to prevent apoptosis during viral infection, is targeted to mitochondria through its short carboxy-terminal region. M11L blocks mitochondrial permeability transition ($\Delta\Psi$m loss) that occurs following a death stimulus. Mutation of the mitochondrial targeting signal of M11L prevents mitochondrial localization and eliminates its antiapoptotic activity (Everett et al., 2000). Thus far, it is not clear whether M11L exerts its protective function independently or by interacting with other proteins on mitochondria.

Cytomegalovirus (CMV) encodes the protein vMIA (viral mitochondrial inhibitor of apoptosis), a product of the immediate early gene UL37 exon 1. vMIA suppresses apoptosis triggered by diverse stimuli by blocking permeabilization of the mitochondrial outer membrane (Goldmacher, 2002). It is predominantly localized in mitochondria, where it appears to form a complex with the adenine nucleotide translocator (ANT). The ANT is an inner membrane component of mitochondrial transition pore complex and has been suggested to act in regulating mitochondrial events during apoptosis and as a target of pro-apoptotic mechanisms. Intriguingly, despite its mitochondrial localization and ANT interaction, vMIA presumably works by a mechanism distinct from Bcl-2 as vMIA neither shares homology with the Bcl-2 family nor binds to Bax and VDAC, properties ascribed to cellular Bcl-2 and Bcl-xL, although the in vivo relevance of these interactions is yet unclear. Thus, vMIA may represent a distinct class of cell death inhibitor.

## Caspase and Granzyme B Inhibitors

Caspases are a family of cysteine proteases and are the principal executioners of apoptosis. All caspases are produced as inactive proenzymes and must

be activated through cleavage to release their active subunits. During cell death, initiator caspases such as caspase-8 are activated by autoprocessing. These activated initiator caspases cleave the precursor form of the more downstream effector caspases such as caspase-3, leading to a loosely defined cascade of caspase activation. Once activated, they cleave a variety of cellular substrates at one or two sites with high specificity (Thornberry and Lazebnik, 1998). Cleavage of cellular substrates by caspases either inactivates their normal function or releases fragments that can further promote cell death. Therefore, caspases are obvious targets for apoptosis inhibition in viral infections. Indeed, the naturally occurring caspase inhibitors were first discovered in the genomes of viruses, including P35 and IAP (inhibitor of apoptosis) of baculoviruses. Moreover, these studies led to the discovery of the cellular IAP protein family that regulates many complex cellular events in addition to apoptosis.

Pox viruses are the only family of viruses encoding serpins (serine protease inhibitors), but unlike cellular serpins, pox virus serpins also inhibit caspases. For example, the CrmA protein of cowpox virus is a specific and potent inhibitor of immunity-modulating caspases such as caspase-1 (ICE) and caspase-8, -4, and -5. Like other serpins, CrmA has been shown to serve as a suicidal pseudosubstrate that covalently inhibits proteases. After binding to the protease, serpins undergo a springlike movement from an initial metastable state to a final hyperstable form, causing distortion of the active site and irreversible inhibition of protease activity (Huntington and Carrell, 2001; Huntington et al., 2000). Caspase-1 is responsible for cleaving the pro-form of IL-1β and IL-18 to release these active pro-inflammatory cytokines. Mice deficient in caspase-1 fail to produce IL-1β and are protected from LPS-induced mortality. Thus, the virus uses this potent modulator of the immune response to greatly reduce the inflammatory response triggered by this cytokine. Overexpressed CrmA inhibits caspase-8, thereby protecting cells from apoptosis induced by TNF, FasL, and, to a less extent, granzyme B (Turner et al., 1999). However, the role of CrmA in blocking apoptosis during pox virus infections is unclear, but CrmA clearly plays an important role in regulating the host inflammatory response.

Another potent caspase inhibitor encoded by baculoviruses is P35 (not to be confused with p53). P35 is a broad-spectrum caspase inhibitor and is required for efficient late viral gene expression and progeny production (Clem et al., 1991; Clem and Miller, 1994). Caterpillars infected with baculoviruses literally melt away as the virus takes over. However, caterpillars infected with P35 mutants are resistant to disease because the virus fails to replicate efficiently. Thus, baculovirus infection of insects is perhaps the clearest example of how inhibition of programmed cell death modulates viral pathogenesis. There is little doubt that equivalent roles for programmed cell death will be uncovered in human disease, but the processes are much more complex. Overexpression of baculovirus P35 blocks cell death in

phylogenetically diverse organisms including mammals in response to many death stimuli. The structure of P35 in a complex with caspase-8 reveals that a covalent thioester linkage between P35 and the caspase active site is stabilized by a postcleavage conformational change in P35 (Xu et al., 2001). Comparing the activity and specificity of P35 to initiator versus effector caspases reveals that P35 more potently inhibits effector caspases such as caspase-3. Some of this specificity might be conferred by slight differences between the structures of initiator and effector caspases (Eddins et al., 2002). Interestingly, although P35 is a potent inhibitor of caspase-9 in vitro, it fails to efficiently block caspase-9 activity in cells or in an animal model (Ryan et al., 2002), suggesting that its function may be dependent on cellular context. So far, there is still no known homologue of P35 outside baculoviruses.

IAP proteins were first identified in baculoviruses because this type of gene could functionally substitute for the P35 gene in the baculovirus AcMNPV. Of the three known baculovirus iap genes (Cp-iap, Op-iap, and Ac-iap), only two of them (Cp-iap and Op-iap) have antiapoptotic activity. Intriguingly, they both exist in strains that lack P35 gene, whereas AcMNPV, the strain encoding P35, has Ac-iap, which lacks antiapoptotic function. Mutagenesis studies of these IAP proteins indicate that the N-terminal BIRs (baculovirus IAP repeats) and the C-terminal RING finger domains are important for their antiapoptotic functions (Clem and Miller, 1994). The only other IAP homologue found in viruses is the pA224L protein of African swine fever virus, although less is known about this protein (Neilan et al., 1997).

Shortly after the identification of baculovirus IAPs, cellular IAP proteins were identified in genomes of humans, *Drosophila*, nematodes, and yeast as defined by the presence of one to three BIR motifs each with a single zinc finger (Salvesen and Duckett, 2002). Until now, eight human IAP-related proteins have been found: c-IAP1, c-IAP2, XIAP, ILP-2, ML-IAP, NAIP, survivin, and Apollon, the human homolog of Bruce (Bir repeat ubiquitin-conjugating enzyme). Not every IAP protein has the ability to inhibit apoptosis. The subset of BIR-containing proteins that lack the C-terminal RING finger is referred to as BIRPs (BIR-containing proteins). New functions of IAP family members continue to be uncovered. Although some of their functions may ultimately be linked to regulation of cell death, other newly assigned functions including cell cycle control, signal transduction, and protein degradation may be unrelated to apoptosis.

Among all the human IAP proteins, XIAP has the strongest activity to inhibit certain caspases (caspase-3, -7, and -9). Close examinations of the molecular and structural basis of XIAP revealed that two separate domains of XIAP are responsible for the suppression of caspase-9 and caspase-3/7. A pocket in the third BIR domain, BIR3, binds to the N-terminus of the caspase-9 small subunit, which is exposed only after cleavage between the large and small subunits. A short stretch of amino acids immediately preceding BIR2 motif of XIAP binds in the active site of caspase-3/7 (Chai et al., 2001; Huang

et al., 2001; Riedl et al., 2001). These two views of how BIR domains inhibit caspases may mean that both mechanisms actually occur within a single BIR domain. In contrast to the covalent inhibitor binding we saw in the case of CrmA and P35, the inhibition of caspases by XIAP is the classical reversible noncovalent binding, but surprisingly the amino-carboxy orientation of XIAP in the caspase active site is opposite to that of the caspase substrates (Stennicke et al., 2002).

Therefore, there are multiple mechanisms for inhibiting caspases employed by both viruses and host cells. Although caspases seem to be a convenient and logical target for viruses to inhibit cell death, few viruses are known to encode direct inhibitors of caspases. There are no P35 homologues in other viruses, the link between CrmA and cell death regulation is tenuous, IAP proteins came from insect viruses and may have additional functions in the virus life cycle. Is the paucity of direct caspase inhibitors in viral genomes explained by the pressure from coevolution? Mammalian cells contain various caspases that are exquisitely and distinctly regulated upon receiving death stimuli, leading to a massive expansion in the activation of caspases. Furthermore, in other situations at least some of these proteases likely carry out many functions not directly related to cell death. Thus, the virus has a challenge to economically block all the caspases that will become activated during cell death without perturbing required functions. So it is perhaps not surprising that most viruses have evolved to target the upstream caspases, thereby blocking the cascade from the top. Viral IAP proteins may competitively inhibit caspases but they do so weakly, suggesting that their sole purpose may not be caspase inhibition or even cell death regulation. It will be easier for us to appreciate these tricks when more is learned about the control of caspases in cell death as well as their other potential physiological functions.

An important arm of the immune system in fighting off viruses is the killing of virus-infected cells by cytotoxic lymphocytes. This is accomplished when lymphocytes activate the Fas death receptor pathway discussed above, but also when lymphocytes deliver granule components to target cells. The serine proteases granzyme A and granzyme B are key components of lymphocyte granules, and when delivered to virus-infected target cells, these proteases facilitate programmed cell death. Though the mechanisms are still unclear, granzymes may induce a caspase-independent death pathway in part because granzyme B cleaves some of the same substrates that are cleaved by caspases. In addition, the granzyme and caspase pathways may converge as granzyme B can also cleave and activate caspases. The L4-100K adenovirus assembly protein was recently discovered to be a potent inhibitor of granzyme B (Andrade et al., 2001). L4-100K is abundantly produced in virus-infected cells and protects these cells from death induced by cytotoxic granules. The other apoptosis inhibitors encoded by adenovirus cannot

substitute for the L4-100K function. Therefore, adenovirus encodes multiple mechanisms for controlling the host response to infection.

## Inhibitors of p53 Link Apoptosis and Cell Cycle Deregulation

As an essential gatekeeper in cell cycle progression, p53 also induces apoptosis primarily through its transactivation or suppressive effects on cellular transcription. Many genes involved in apoptotic pathways are up- or down-regulated by p53, including the well-known pro-apoptotic genes such as Fas, Apaf-1, Bax, and several BH3-only proteins such as PUMA and NOXA. The level of p53 protein in a cell is normally low because of its short half-life. However, p53 is readily activated and stabilized during DNA damage, oncogene activation, or other abnormal cell cycle progression (Hickman et al., 2002). Replication of some viruses requires host cells to enter the cell cycle. The aberrant growth signals from viral proteins in many cases lead to p53 mediated apoptosis. Therefore, many viruses have devised mechanisms to override p53-induced cell death.

Adenovirus E1A protein has been known for a long time to stimulate cell proliferation and induce cell death through Rb and p53, which will be discussed later in this chapter. To balance the death-inducing effects of E1A and win time for proper replication, adenoviruses have evolved another three genes that inhibit the apoptosis induced by E1A. E1B-19K blocks apoptosis at the mitochondrial level as mentioned earlier; E1B 55K and E4orf6 independently bind to p53 at the N- and C-terminus, respectively, and inhibit the transcription activity of p53 (Dobner et al., 1996; Sarnow et al., 1982; Yew and Berk, 1992). Furthermore, E1B-55K and E4orf6 also collaborate to target p53 for ubiquitination and degradation by forming a multiprotein complex containing a novel E3 ubiquitin ligase (Querido et al., 1997, 2001). Similarly, the large T antigen encoded by SV40 binds to and inactivates p53 (Mietz et al., 1992), while human papillomavirus (HPV) E6 protein binds p53 and targets it for degradation via the ubiquitin-proteosome pathway (Scheffner et al., 1990). Other large DNA viruses also target p53. The beta herpes virus, human cytomegalovirus (CMV) immediate early gene IE2, has been linked to p53 targeting and cell cycle regulation (Tsai et al., 1996).

## Viral Cell Death Inhibitors That Stimulate Cellular Survival Signaling

The PI3 kinase-Akt signaling pathway is a major pathway in relaying survival signals from cell surface growth factor receptors. Recruitment and activation of PI3K by tyrosine kinase receptors generate phosphoinositide phosphates PIP2 and PIP3 at the inner side of plasma membrane. The pleck-

strin homology (PH) domain of Akt then binds to these phospholipids and translocates to the plasma membrane where it is activated by phosphoinositide-dependent kinase (PDK1)-mediated phosphorylation (Brunet et al., 2001; Datta et al., 1999).

Several substrates of activated Akt have been identified and shown to promote cell survival. Akt directly phosphorylates forkhead box transcription factors and inactivates their capacity to upregulate death genes, such as FasL and Bim. Pro-apoptotic Bcl-2 family protein Bad can be phosphorylated by Akt, and then sequestered by 14-3-3 in the cytosol to inhibit its death-promoting functions. Further phosphorylation in the BH3 domain of Bad by other kinases is important for the release of antiapoptotic Bcl-xL from Bad (Datta et al., 1997, 2000; del Peso et al., 1997). The link between Akt and Bad phosphorylation thus provides a mechanism for viruses to directly modulate apoptosis at the mitochondria level, preventing the amplification phase of the caspase cascade. Akt also indirectly inhibits the activity of p53, activates CREB and NF-κB to increase the expression of survival genes such as Bcl-xL and IAP, and may phosphorylate caspase-9 under some circumstances. Akt has also been suggested to promote cell survival through control of metabolic processes and repression of GSK3 (glycogen synthase kinase-3).

The middle T antigen (mTAg) of polyoma virus interacts with tyrosine kinases and PI3K, and has been shown to activate Akt in a PI3K-dependent manner (Dahl et al., 1998; Meili et al., 1998; Summers et al., 1998). Thus, the PI3K-Akt pathway is presumed to contribute to the transforming ability of mTAg and tumorigenic activity of polyoma virus. A recent study of HIV revealed another strategy to inhibit cell death by phosphorylating Bad. The Nef protein of HIV activates PI3K, leading to activation of another kinase, p21-activated kinase (PAK), which then phosphorylates Bad and inhibits apoptosis (Wolf et al., 2001). Therefore, Nef inhibits death by at least two means, directly associating and blocking ASK1 activation as discussed earlier, and by activating PI3K to eventually target Bad. Another multifunctional protein of HIV, Tat, also has a role in the activation of PI3K (Borgatti et al., 1997; Deregibus et al., 2002), as does HBx protein of hepatitis B virus (Lee et al., 2001). In addition, antiapoptotic signaling and NF-kappaB activation in respiratory syncytial virus (RSV) infections involve activation of PI3K-dependent pathways. Blockade of PI3K in this case results in rapid, premature apoptosis (Thomas et al., 2002). Undoubtedly, many more viral proteins will be found to regulate cellular survival pathways.

## Induction of Apoptosis by Viruses

Cell death in viral infections can be induced directly by specific viral proteins or through indirect mechanisms as the consequence of disrupting

cellular functions. The broad array of antiapoptotic strategies employed by virtually all virus families validates the proposed role for apoptosis as a host defense mechanism against viruses. However, apoptotic responses to viruses can also be detrimental to the host organisms, as in cases where the virus takes advantage of this pathway to facilitate invasion, or when the apoptotic response enhances the pathogenesis. We will review here some of the mechanisms of virus-induced apoptosis and cell death, although in general this aspect of virology is less well understood.

## Adenovirus

E1A is a multifunctional protein and the first viral gene to be transcribed after adenovirus infects a cell. E1A is a transcription factor that activates transcription of other viral genes to facilitate viral replication. E1A also binds to cellular proteins that negatively regulate cell growth, including the retinoblastoma protein (Rb) and transcription coactivator p300. These and other activities of E1A serve to deregulate cell cycle control and promote a gene expression pattern that is suitable for productive virus infection and primary cell transformation. However, shortly after these cells begin to divide, they die. This pro-death function of E1A maps to its Rb- and p300-binding sites (White, 2001).

Rb is a cell cycle checkpoint protein that normally binds to and inhibits the transcription factor E2F from driving cells into S phase. Phosphorylation of Rb by cyclin-dependent kinases causes Rb to release E2F or other E2F family proteins and associated factors. E2F induces a variety of genes involved in S phase progression and upregulates p14ARF, a protein that binds and neutralizes the activity of MDM2. MDM2 functions by targeting p53 for proteolysis via the ubiquitin pathway to maintain the normally low level of p53 in a cell. Therefore, inhibition of MDM2 by p14ARF results in the stabilization of p53 and contributes to p53-dependent apoptosis induced by E1A. Inhibition of p300 by E1A also leads to a rise in p53 levels and apoptosis. Yet this is not the only way that E1A can induce apoptosis since p53-null cells are still susceptible to E1A-induced apoptosis, and we do not yet fully understand the consequences of E1A binding to Rb and p300. One recent report shows that E1A indirectly triggers proteosome-dependent degradation of the cellular apoptosis inhibitor cFLIP, which results in sensitization of infected cells to TNF-induced apoptosis (White, 2001). E1A also affects downstream events in the death pathway. After double knockout cells lacking both of the pro-apoptotic Bcl-2 family proteins Bax and Bak were utilized, it was reported the death signaling initiated by E1A is dependent on Bax and Bak. Thus, E1A-induced apoptosis ultimately goes through the same mitochondrial apoptosis pathway that is used by many other death stimuli (Cuconati et al., 2002; Degenhardt et al., 2002).

Adenovirus E4orf4, a 14 kD polypeptide that may play multiple roles during adenovirus infection, has been implicated in the selective killing of transformed cells through a p53-independent cell death mechanism (Branton and Roopchand, 2001). When overexpressed in mammalian cells, E4orf4 localizes to the nucleus, cytoplasm, and plasma membrane. At least two distinct mechanisms of cell death are likely to be initiated from these different cellular compartments, and both pathways appear not to require the participation of caspases. Cytoplasmic E4orf4 induces a Src kinase-mediated apoptotic signal requiring the tyrosine phosphorylation of E4orf4, and rapidly leads to membrane blebbing and cell death. Calpain activity is involved in this pathway (Gingras et al., 2002; Lavoie et al., 2000). Nuclear E4orf4, on the other hand, does not require phosphorylation and induces a still mysterious death signal from the nucleus (Robert et al., 2002). Interestingly, E4orf4 was suggested to promote cell death by binding protein phosphatase 2 (PP2A), which has a wide variety of substrates playing roles in cell metabolism, cell cycle and cell growth regulation (Janssens and Goris, 2001). However, the connection between PP2A and either of these two potential mechanisms of E4orf4 killing has not yet become apparent.

## Retrovirus HIV-1

The loss of CD4+ and CD8+ T cells by apoptosis, including both infected and uninfected cells, contributes significantly to the pathogenesis of HIV infection. Despite the enormous amount of work performed over the past decade, the relationship between HIV infection and T cell death remains a very complex issue. We now know that multiple viral genes and various apoptotic mechanisms are involved at different stages for different targets, but little is known about the exact regulation of these switches and what their contributions are to viral infection and disease progression.

Apoptosis induced by HIV-1 is often described as being one of three types (Roshal et al., 2001). "Death from without" refers to a type of extrinsic cell death occurring before or immediately after binding/entry of virus particles to the cell, prior to de novo viral gene expression. One example of this is the ability of the HIV envelope glycoprotein gp120 to cross-link CD4 receptors and/or the chemokine coreceptors on T cells, which primes these cells to Fas-mediated killing. Virus-induced engagement of Fas or TNFα death-receptor-initiated apoptosis also can be assigned to this category. "Death from within" is caused by cellular responses to virus replication or to the expression of individual viral genes capable of inducing apoptosis, such as Tat, Nef, and Vpr. Tat was also shown to kill uninfected cells when secreted from infected cells and taken up directly by uninfected cells. Thus, Tat can induce cell death "in trans."

Besides being famous for its ability to be released from infected cells and target uninfected cells, Tat has also been associated with the upregula-

tion of caspase-8, increased secretion of TNF-α, and inhibition of superoxide dismutase. However, the mechanisms by which Tat sensitizes cells to death receptor signaling leading to caspase activation, and how Tat alters the cellular redox status toward oxidation are not understood (Li et al., 1995; Westendorp et al., 1995a, 1995b). Nef enhances the expression of both Fas and FasL (Xu et al., 1999; Zauli et al., 1999), whereas Vpr has not only been implicated in G2/M cell-cycle-arrest-related cell death, but also directly affects mitochondrial permeability (Jacotot et al., 2000; Stewart et al., 1997). However, these very same HIV proteins can also inhibit apoptosis at least at early stages of infection. Thus, we are still far away from understanding the regulation of HIV-induced apoptosis, and the role of HIV-induced apoptosis in the pathogenesis of AIDS is even further beyond our grasp.

## Sindbis Virus

Sindbis virus is a positive-sense single-stranded RNA alphavirus that is transmitted by mosquitoes in nature and has been used for decades in a mouse model of viral encephalomyelitis where Sindbis virus is profoundly neuronotropic. In newborn mice, Sindbis virus causes apoptosis in neurons of the central nervous system, which is believed to directly correlate with mouse mortality (Lewis et al., 1996). Interestingly, although Sindbis virus readily induces apoptosis in a variety of mammalian cell lines and in the mouse brain, its ability to induce apoptosis in mosquito cells and in mosquitoes is severely impaired. Sindbis virus kills newborn mice but does not kill mosquito larvae despite efficient replication, consistent with the obligatory role of mosquitoes in completing the virus life cycle (Karpf and Brown, 1998). Thus, it appears that neuronal death induced by Sindbis virus is a major pathogenic factor instead of being an important host defense mechanism, especially when we consider that it has been shown the virus replicates efficiently in apoptotic neurons. One interesting feature of Sindbis infection is the age-dependent susceptibility of the host. Immature neurons are killed by both avirulent and virulent strains, whereas older mice and more mature neuron cultures become resistant to cell death induced by the avirulent strains (Griffin et al., 1994; Lewis et al., 1996). This indicates that there is an age-dependent regulation of cell death in the mouse central nervous system that suppresses neuronal cell death as the animal matures. Recently, a study using GeneChip technology to compare mock or infected neonatal and weanling mouse brains revealed developmental regulation of numerous genes, including some apoptosis regulatory genes, consistent with age-dependent susceptibility. Proapoptotic factors caspase-3 and TRAF4 were downregulated, and one inflammatory response gene, ISG12, was upregulated in brains of 4-week-old mice compared to 1-day-old mice following infection with Sindbis virus (Labrada et al., 2002).

Taking advantage of the ability of Sindbis virus to act as both an expression vector and a death stimulus, various cellular apoptosis regulators have been tested for their potential roles in Sindbis virus infections (Hardwick and Levine, 2000). Both Bcl-2 and Bax are capable of inhibiting apoptosis in Sindbis-infected neurons even though they are thought to have opposite functions in many other cases (Levine et al., 1993; Lewis et al., 1999). A neurovirulent strain of Sindbis virus, NSV, can induce death of mature neurons in older animals. NSV harbors a point mutation in the E2 glycoprotein that is involved in the early events of virus entry but also plays a role in replication. The intracellular events prior to initiation of the caspase cascade that lead to Sindbis-virus-induced cell death are not understood. Interestingly, antagonists of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors have a small but significant protective effect against Sindbis-virus-induced death in neurons. These findings are consistent with the idea that Sindbis virus infection of neurons may trigger excitatory death pathways and cause damage not only to infected neurons but also to bystander uninfected neurons (Kerr et al., 2002; Nargi-Aizenman and Griffin, 2001).

## Other Viral Factors Involved in Cell Death

The chicken anemia virus protein Apoptin is a nonstructural protein that has been shown to induce apoptosis in a large number of transformed and tumor cell lines, but not in normal diploid cells. This appealing feature makes Apoptin a good candidate anticancer agent. Unlike many apoptotic stimuli such as irradiation or chemotherapy agents that are most effective in cells possessing a functional p53 and are inhibited by Bcl-2, Apoptin acts independently of p53, and the pro-death activity of Apoptin can be enhanced by Bcl-2 (Pietersen and Noteborn, 2000). The mechanism of Apoptin-induced apoptosis is still under investigation.

A new pro-apoptotic protein was recently identified in influenza virus A. This 87–90 amino acid protein, PB1-F2, is encoded by an alternative reading frame in the gene for one of the three viral polymerase subunits (Chen et al., 2001). Influenza viruses that have maintained this open reading frame are more efficient at killing infected cells, and viruses with targeted mutations that interfere with PB1-F2 expression have a reduced capacity to induce death. PB1-F2 localizes to mitochondria and causes a loss of mitochondrial membrane potential. Like HIV Vpr, PB1-F2 can form pores in membranes. Expression of PB1-F2 by influenza virus infection, by microinjection or transfection of a plasmid encoding PB1-F2, or by simply adding the PB1-F2 protein to cells induces extensive apoptosis in a cell-type-dependent manner (Chen et al., 2001). Thus, PB1-F2 can kill cells in both *cis* and *trans*. Again, the mechanisms of action or the role of PB1-F2 in pathogenesis is not known. Further understanding of PB1-F2 may help us to conquer this widely distributed pathogen of humans and animals.

## Conclusion

From the above examples of how various viruses inhibit or induce apoptosis, it is obvious that apoptosis is a dual-blade sword. While preventing virus replication or spread in the host, it is also responsible for the loss of functional cells to the detriment of the host. Research into the mechanisms by which viruses regulate apoptosis has provided an abundance of information about how both viruses and cells initiate and proceed through the suicidal execution, but there remain many gaps in both the details and especially in mechanisms of disease pathogenesis. We still do not know how most viruses induce programmed cell death, and much more needs to be learned about the surveillance system normally running in a cell to sense changes upon viral infection. Besides the well-known death receptor and mitochondrial pathways, there are likely to be other yet undiscovered mechanisms of virus-induced cell death.

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CHAPTER

18

# CELL DEATH IN CANCER AND CANCER THERAPY

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Apoptosis or programmed cell death is a distinct, intrinsic cell death program that occurs in various physiological and pathological situations (Hengartner, 2000). Apoptosis is a key regulator of tissue homeostasis, which critically depends on the balance between proliferation and cell death (Evan and Vousden, 2001). One of the most important recent advances in cancer research is the recognition that apoptosis plays a major role in both tumor formation and treatment response (Johnstone et al., 2002; Lowe and Lin, 2000; Reed, 1999; Herr and Debatin, 2001; Kaufmann and Gores, 2000). The realization that apoptosis is a gene-directed program implies that it can be disrupted by genetic mutations (Johnstone et al., 2002). Some oncogenic mutations block apoptosis, leading to tumor initiation and progression (El-Deiry, 1997). To this end, failures in apoptosis pathways may create a permissive environment for genetic instability and accumulation of gene mutations, promote resistance to immune-based destruction, facilitate growth-factor- or hormone-independent survival, and support anchorage-independent survival during metastasis (Igney and Krammer, 2002). Conversely, other oncogenic changes such as the myc oncogene can promote apoptosis, thereby producing selective pressure on tumor cells to override apoptosis during multistage carcinogenesis (Evan and Vousden, 2001). In addition, killing of tumor cells by diverse cytotoxic approaches, such as

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anticancer, drugs, γ-irradiation, suicide genes, or immunotherapy, has been shown to be mediated through induction of apoptosis in target cells (Kaufmann and Earnshaw, 2000; Herr and Debatin, 2001). Since the same oncogenic alterations and defects in apoptosis programs that suppress cell death during tumor development can also confer resistance to cytotoxic therapies, apoptosis provides a conceptual framework to link cancer formation and cancer therapy. Thus, elucidation of the core machinery of the cell death pathway has provided new insight into cancer biology, revealing novel strategies for cancer therapy.

## Mechanisms of Apoptosis

### Caspases as Central Death Effector Molecules

Most apoptosis signaling pathways ultimately result in activation of caspases, a family of cysteine proteases that act as common death effector molecules in various forms of cell death (Thornberry and Lazebnik, 1998; Earnshaw et al., 1999; Los et al., 1999). Twelve human caspases with different substrate specificity have so far been identified that cleave next to aspartate residues. Caspases are synthesized as inactive zymogens and activated by proteolytic cleavage, for example, cleavage between the large and the small subunit followed by cleavage between the large subunit and the prodomain. The fact that active caspases can activate each other by cleavage at identical sequences results in amplification of activity through a protease cascade.

Caspases involved in apoptosis signaling are categorized into initiator and effector caspases, respectively (Thornberry and Lazebnik, 1998). Initiator caspases transduce various signals into protease activity and are directly linked to death-inducing signaling complexes (DISCs): Caspase-8 or -10 via their death effector domain (DED) interact with adaptor proteins (FADD) recruited and bound to activated death receptors, while caspase-9 is recruited to the apoptosome via its CARD domain. Effector caspases cleave various cytoplasmic or nuclear substrates, marking many of the morphologic features of apoptotic cell death (Hengartner, 2001). For example, polynucleosomal DNA fragmentation is initiated by cleavage of ICAD (inhibitor of caspase-activated Dnase), the inhibitor of the endonuclease CAD (caspase-activated DNase) that cleaves DNA into the characteristic oligomeric fragments (Hengartner, 2001). DNA condensation is caused by AIF, a mitochondrial protein that translocates to the nucleus upon death triggering, and by Acinus, which stands for "apoptotic chromatin condensation inducer in the nucleus" (Ferri and Kroemer, 2000). AIF may also mediate caspase-independent cleavage of DNA into larger fragments (Daugas et al., 2000). Likewise, loss of overall cell shape is due to proteolysis of cytoskeletal proteins including fodrin, gelsolin, actin, plectrin, and cytokeratin,

while nuclear shrinking and budding occur after degradation of lamin (Hengartner, 2001).

### Pathways of Caspase Activation

Activation of caspases can be triggered principally by two different mechanisms (Fig. 1): According to the induced proximity model initiator, caspases such as caspase-8 or -9 are activated in a multimeric complex, for example, caspase-8 in the death-inducing signaling complex (DISC) and caspase-9 within the apoptosome (Walczak and Krammer, 2000; Schulze-Osthoff et al., 1998; Krammer, 2000; Fulda and Debatin, 2002b). Alternatively, caspases are activated by catalytic processing of the zymogens at specific cleavage sites (Thornberry and Lazebnik, 1998). Caspase activation can be initiated through different entry sites, for example, at the plasma membrane by death-receptor-mediated signaling (receptor pathway) or at the mitochondria (mitochondrial pathway) (Fulda and Debatin, 2002b). Stimulation of death receptors of the tumor necrosis factor (TNF) receptor superfamily such as CD95 (APO-1/Fas) or TRAIL receptors results in receptor aggregation and recruitment of the adaptor molecule Fas-associated death domain (FADD) and caspase-8 to form the DISC (Scaffidi et al., 1998; Walczak and Krammer, 2000). Upon recruitment, caspase-8 becomes activated and initiates apoptosis by direct cleavage of downstream effector caspases. The mitochondrial pathway is initiated by the release of apoptogenic factors such as cytochrome c, apotosis-inducing factor (AIF), Smac/Diablo, Omi/HtrA2, endonuclease G, caspase-2 or -9 from the mitochondrial intermembrane space (Kroemer and Reed, 2000; Constantini et al., 2000). The release of cytochrome c into the cytosol triggers caspase-3 activation through formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex. Smac/Diablo and Omi/HtrA2 promote caspase activation by neutralizing the inhibitory effects to IAPs, while AIF and endonuclease G cause DNA condensation (Li et al., 2001; Martins, 2002; Daugas et al., 2000; Du et al., 2000).

The receptor and mitochondrial pathway can be interconnected at different levels (Roy and Nicholson, 2000). Following death receptor stimulation, activation of caspase-8 may result in cleavage of Bid, a BH3-domain-containing protein of the Bcl-2 family that assumes cytochrome-c-releasing activity upon cleavage, thereby initiating a mitochondrial amplification loop (Roy and Nicholson, 2000). In addition, mitochondria-triggered caspase-6 cleavage may feed back to the receptor pathway by cleaving caspase-8 (Slee et al., 1999).

## Apoptosis and Cancer

The accumulation of neoplastic cells can occur through enhanced proliferation, diminished cell turnover, or both (Evan and Vousden, 2001). The

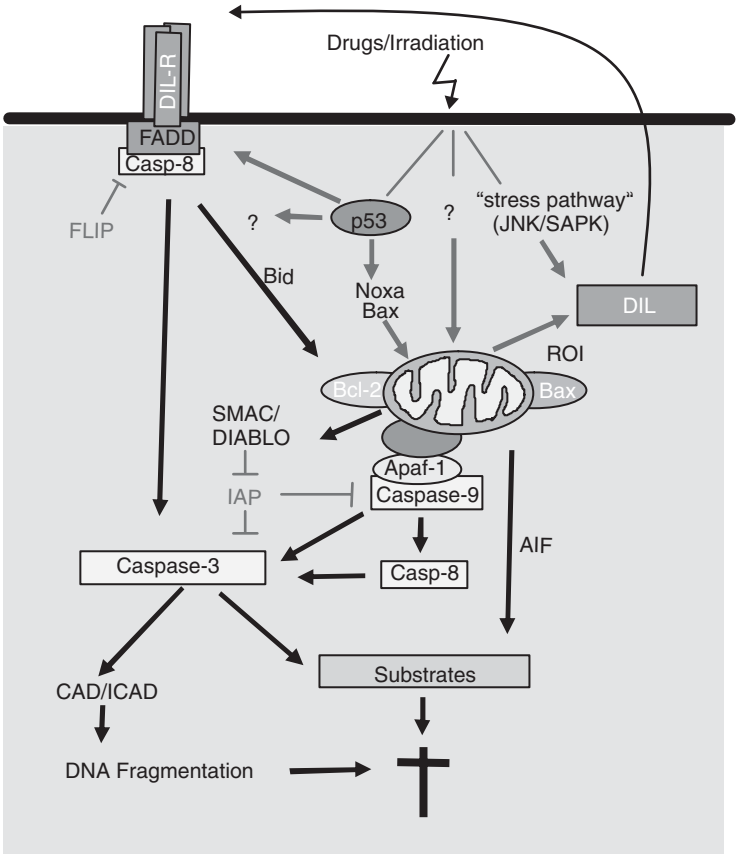


FIGURE 1. Activation of apoptosis pathways by anticancer therapy. Apoptosis pathways can be initiated through different entry sites, for example, at the plasma membrane by death-receptor-mediated signaling (receptor pathway) or at the mitochondria (mitochondrial pathway). Stimulation of death receptors of the tumor necrosis factor (TNF) receptor superfamily (DIL-R) such as CD95 (APO-1/Fas) or TRAIL receptors by death-inducing ligands (DIL) results in receptor aggregation and recruitment of the adaptor molecule Fas-associated death domain (FADD) and caspase-8. Upon recruitment, caspase-8 becomes activated and initiates apoptosis by direct cleavage of downstream effector caspases. The mitochondrial pathway is initiated by the release of apoptogenic factors such as cytochrome c, apoptosis-inducing factor (AIF), or Smac/Diablo from the mitochondrial intermembrane space. The release of cytochrome c into the cytosol triggers caspase-3 activation through formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex. Smac/Diablo promotes caspase activation by neutralizing the inhibitory effects to IAPs, while AIF causes DNA condensation. The receptor and mitochondrial pathway can be interconnected at different levels, for example, by Bid, a BH3-domain-containing protein of the Bcl-2 family that assumes cytochrome-c-releasing activity upon cleavage by caspase-8. Activation of caspases is negatively regulated at the receptor level by FLIP that block caspase-8 activation, at the mitochondria by Bcl-2 family proteins, and by inhibitor of apoptosis proteins (IAPs). See the text for further details.

importance of apoptotic cell death in the control of the carcinogenic process has been suggested by studies of colon specimens harvested at various times along the adenoma-to-carcinoma transition (Lowe and Lin, 2000). These studies revealed that the rate of apoptosis is relatively high in normal colonic epithelium, intermediate in adenomas, and low in carcinomas. The potential contribution of reduced cell death to tumor development was first established, when the Bcl-2 oncogene was identified. Bcl-2 did not behave like a typical oncogene, since instead of disturbing the control of proliferation, Bcl-2 promotes cell survival by blocking apoptosis (Antonsson and Martinou, 2000). Bcl-2 was first identified at the chromosomal breakpoint of the t(14;18) translocation in a human leukemia cell line, a translocation commonly found in follicular lymphoma leading to deregulated expression of the Bcl-2 protein (Tsujimoto et al., 1984). Alterations in the expression of antiapoptotic or pro-apoptotic members of the Bcl-2 family proteins have been described in various human cancers (Reed, 1999). Some of these mechanisms involve structural gene alterations, for example, single nucleotide substitution or frameshift mutations that inactivate the Bax gene in certain types of colon cancer and hematopoetic malignancies (Reed, 1999). However, in most cases aberrant expression of Bcl-2 proteins is regulated at the transcriptional or posttranscriptional level. Imbalances in the ratio of anti- and pro-apoptotic Bcl-2 proteins favor tumor cell survival instead of cell death (Antonsson and Martinou, 2000). Also, Bcl-2 can cooperate with oncogenes such as the c-myc oncogene in the process of multistep carcinogenesis by blocking c-myc-induced apoptosis (Evan and Vousden, 2001).

p53 was the first tumor suppressor gene linked to apoptosis (Vogelstein et al., 2000; Vousden, 2000). p53 mutations occur in the majority of human cancers and are often associated with advanced disease and poor prognosis (Wallace-Brodeur and Lowe, 1999). p53 functions as a checkpoint protein involved in cell cycle arrest, DNA repair, and apoptosis (Vogelstein et al., 2000; Vousden, 2000). Studies using p53 knockout mice showed that p53 was required for radiation-induced apoptosis in the thymus (Lowe et al., 1993). In addition to p53 mutations, several upstream or downstream components of the p53 pathway are disrupted in human tumors (Vogelstein et al., 2000; Vousden, 2000). For example, amplification of Mdm-2, which antagonizes p53 through proteasome-mediated degradation of p53, or loss of the tumor suppressor gene INK4a/ARF, which activates the p53 pathway, for example, during oncogene-induced apoptosis, similarly results in disruption of the p53 pathway of apoptosis. Likewise, alterations in p53 effectors such as caspase-9, Apaf-1, or Bax have been shown to promote oncogenic transformation and tumor development in mouse tumor models.

In addition to the studies on Bcl-2 and p53 that established the importance of apoptosis in tumor formation, mutations in many apoptosis-related genes have subsequently been demonstrated to contribute to carcinogenesis (Lowe and Lin, 2000). Given the central role of caspases for cell death exe-

cution, one might expect a high frequency of caspase mutations in tumors. Interestingly, however, screening for mutations in initiator or executioner caspases in a variety of human tumors has not revealed a high frequency of genomic aberrations in caspase genes (Mandruzzato, 1999; Teitz, 2000). Instead, caspase expression and function may be impaired by epigenetic alterations such as promoter hypermethylation (Teitz et al., 2000). To this end, caspase-8 expression was found to be frequently inactivated by hypermethylation of regulatory sequences of the caspase-8 gene in a number of different tumor cells derived from neuroblastoma, malignant brain tumors, Ewing tumor, and small lung cell carcinoma both in vitro and also in vivo in primary tumor samples (Teitz et al., 2000; Fulda et al., 2001b).

Several antiapoptotic alterations can disrupt signaling through the death receptor pathway. First, mutations of the CD95 receptor, which normally controls cell numbers in the immune system by eliminating cells through apoptosis, can lead to lymphoproliferative disorders and malignant lymphatic disease (Debatin, 1998). Also, death receptor expression may vary between different cell types and can be downregulated in tumor cells, for example, CD95 in hepatomas compared to normal hepatocytes, which has been assumed to contribute to the escape of tumor cells from negative growth control (Igney and Krammer, 2002). Moreover, overexpression of decoy receptors such as DcR3, which binds CD95 ligand, in colon or lung carcinoma may help tumor cells to avoid the lethal signals after death receptor stimulation (Krammer, 2000). Signaling by death receptors can be negatively regulated by proteins that associate with their cytoplasmic domains, for example, SODD, or by proteins such as FLIP that prevent the interaction between the adaptor molecule FADD and pro-caspase-8 (Igney and Krammer, 2002; French, 2002). High FLIP expression found in many tumor cells has been correlated with resistance to CD95- and TRAIL-induced apoptosis (Tschopp et al., 1998). Importantly, FLIP expression was associated with tumor escape from T cell immunity and enhanced tumor progression in experimental studies in vivo, pointing to the role of FLIP as a tumor-progression factor (French and Tschopp, 2002). Thus, inactivation of the death receptor pathway may facilitate carcinogenesis by rendering tumor cells resistant to killing by CD95 ligand-expressing cytotoxic T lymphocytes (French and Tschopp, 2002). Also, tumor cells may become resistant to suicide pathways activated upon suboptimal growth condition or upon detachment from the extracellular matrix, which involve death receptor signaling (Igney and Krammer, 2002). Alternatively, expression of the cytotoxic CD95 ligands on the cell surface of tumor cells may promote immune escape of tumors via the tumor cell's counterattack against cytotoxic T lymphocytes, which express CD95 receptor and are susceptible to CD95-triggered apoptosis, resulting in an immune privileged environment around the tumor site (Green and Ferguson, 2001).

Inhibitor of apoptosis proteins (IAPs) such as XIAP, cIAP1, cIAP2, survivin, livin, or ML-IAP have emerged as endogenous caspase inhibitors

(Deveraux and Reed, 1999; Holczik and Korneluk, 2001). Interestingly, survivin is expressed at high levels in the majority of human cancers and represents the fourth most common transcriptome of the human genome, indicating that it may contribute to the malignant phonotype of cancer cells (Altieri, 2001; Velculescu et al., 1999). Elevated survivin expression has been associated with poor prognosis in a variety of human neoplasms, for example, neuroblastoma, colon carcinoma, gastric carcinoma, or leukemia (Adida, 1998, 2000; Altieri, 2001). In contrast, low or undetectable levels of survivin are found in most nonproliferating, normal adult tissues (Altieri, 2001).

Moreover, mounting evidence suggests that perturbations of the PI3K/Akt pathway play a central role in tumorigenesis (Datta et al., 1999; Blume-Jensen and Hunter, 2001). The PI3K/Akt pathway can be altered at a variety of steps in tumor cells. As a result of production of autocrine growth factors, elevated levels of growth factor receptors, or constitutively active, mutated receptors, enhanced signaling from receptor tyrosine kinases can occur, for example, in breast carcinoma as a result of HER2/neu overexpression (Blume-Jensen and Hunter, 2001). Alternatively, the gene for PI3K or for AKT2 is amplified in a subset of ovarian carcinoma (Blume-Jensen and Hunter, 2001). Likewise, absence of the tumor suppressor gene PTEN because of gene loss or mutations, which antagonize the pro-survival function of Akt through dephosphorylation and inactivation of Akt, frequently occurs in several tumors including malignant glioma (Simpson and Parsons, 2001). Also, mutated constitutively active Ras isoforms, which are found in 30% of cancers, in particular in pancreatic carcinoma, or the fusion protein bcr/abl, the transforming kinase of CML, can directly activate PI3K (Downward, 1998). In addition to the diversity of changes, which may result in enhanced signaling through the PI3K/Akt pathway in tumors, numerous substrates for the serine/thronine kinase Akt have been implicated in tumorigenesis (Datta et al., 1999). Among its pleiotropic effects, activated Akt is a well-established survival factor and exerts its antiapoptotic function through transcriptional and posttranscriptional modifications of key molecules involved in apoptosis signaling, for example, Bad, fork head transcription factors, NFκB, or cell cycle regulatory proteins (Datta et al., 1999).

The transcription factor NFkB has been connected with multiple aspects of oncogenesis, including cell proliferation, inhibition of apoptosis, cell cycle, and migration (Mayo and Baldwin, 2000). The t(10,14) chromosomal translocation breakpoint associated with NFκB2 was originally found in a case of B-cell non-Hodgkin's lymphoma and occurs in a number of lymphoid neoplasms (Karin et al., 2002). Multiple human tumors, for example, Hodgkin lymphoma, pancreatic carcinoma, or breast carcinoma, have evolved mechanisms for deregulating the NFκB pathway, suggesting that NFκB is involved in tumorigenesis (Karin et al., 2002). Mutations in the IκBa gene have been detected in Hodgkin's lymphoma, thereby rendering NFκB constitutively active (Karin et al., 2002).

## Apoptosis Pathways in Cancer Therapy

Most chemotherapeutic agents now in clinical use were developed using empirical screens designed to identify agents that selectively or nonselectively kill cancer cells. Studies on drug action initially focused on intracellular drug targets, drug–target interaction, or resistance mechanisms that prevent drug–target interaction. It is now well established, at least in vitro, that the majority of anticancer agents primarily act by triggering apoptosis in tumor cells (Herr and Debatin, 2001; Kaufmann and Earnshaw, 2000). This implies that cellular responses occurring after drug–target interaction have a profound impact on drug-induced cytotoxicity. The underlying mechanisms for initiation of an apoptosis response upon cytotoxic therapy may be different for different stimuli and are only partially understood. However, damage to DNA or to other critical molecules and/or subcellular structures appears to be a common early hit by some inducers, which is then propagated by the cellular stress response (Herr and Debatin, 2001; Rich et al., 2000). Multiple stress-inducible molecules, for example, JNK, MAPK/ERK, NFκB, or ceramide, may have a profound impact on apoptosis pathways (Leppa and Bohmann, 1999; Davis, 2000). On the other hand, cytotoxic T cells or NK cells may release compounds such as granzyme B, which directly activate downstream apoptosis effector mechanisms inside the cell (Herr and Debatin, 2001). Since the cytotoxic effects of current therapies are mediated by apoptosis, disruption of apoptosis signal transduction pathways can reduce treatment sensitivity. Since agents with distinct primary intracellular targets can initiate apoptosis through similar mechanisms, defects in apoptosis programs may produce multidrug resistance.

### Signaling Pathways in Cancer Therapy

Apoptosis in response to cancer therapy proceeds through activation of the core apoptotic machinery including the receptor and mitochondrial signaling pathway (Herr and Debatin, 2001; Kaufmann and Earnshaw, 2000). The relative contribution of the receptor and mitochondrial pathway to drug-induced apoptosis has been a subject of controversy (Herr and Debatin, 2001; Kaufmann and Earnshaw, 2000). Although a number of initial studies suggested that cancer-therapy-triggered apoptosis involves activation of the CD95 receptor/ligand system (Friesen et al., 1996; Muller et al., 1998; Fulda et al., 1998; Debatin, 1999), compelling evidence subsequently indicated that the majority of cytotoxic drugs initiate cell death by triggering the cytochrome c/Apaf-1/caspase-9 dependent pathway through the mitochondria (Eischen et al., 1997; Constantini et al., 2000). To this end, targeted disruption of genes involved in the mitochondrial pathway points to the crucial and indispensable role of this pathway for apoptosis in response to anticancer drug treatment. Caspase-$9^{-/-}$ embryonic stem cells and Apaf-$1^{-/-}$

thymocytes are resistant to cytotoxic drugs, but nevertheless remain sensitive to death receptor triggering (Hakem et al., 1998; Yoshida et al., 1998). In contrast, FADD$^{-/-}$ and caspase-8$^{-/-}$ fibroblasts are refractory to death receptor stimulation, but equally sensitive to cytotoxic drugs, suggesting that this death receptor pathway has a dispensable role in drug-induced apoptosis, at least in nontransformed cells (Yeh et al., 1998; Varfolomeev et al., 1998). However, the relative contribution of the death receptor versus the mitochondrial pathway may depend on the cytotoxic drug, dose, and kinetics, or on differences between certain cell types similar to the cell-type-dependent signaling in the CD95 pathway (Fulda, 2001b). Importantly, this amplification of the chemoresponse through activation of the CD95 system may be clinically meaningful, since it may critically affect the time required for execution of the death program (Tang et al., 2000). The net outcome of signaling through the core apoptotic machinery is regulated by multiple pro- and antiapoptotic signaling paths as discussed below.

## Pro-apoptotic Signaling in Cancer Therapy

Caspases. Given the important role of caspases as effector molecules in various forms of cell death including drug-induced apoptosis, the ability of anticancer agents to trigger caspase activation appears to be a critical determinant of sensitivity or resistance to cytotoxic therapies (Fulda and Debatin, 2002b). As a consequence, inhibition of caspase activation may be an important factor in chemoresistance (Fulda and Debatin, 2002; Faderl and Estrov, 2001; Svingen et al., 2000).

First, expression levels of individual caspases may have an impact on their overall activity, since activation of caspases may simply be impaired by deficient expression levels of caspases (Teitz et al., 2000; Fulda et al., 2001a,b; Estrov et al., 1998; Koomagi and Volm, 2000). For example, MCF-7 breast carcinoma cells completely lack caspase-3 expression due to a frameshift mutation within exon 3 of the caspase-3 gene (Janicke et al., 1998). These cells can be sensitized by transfection of pro-caspase-3 toward treatment with cytotoxic drugs (Yang et al., 2001). Next, caspase expression may be impaired by epigenetic alterations such as promoter hypermethylation (Teitz et al., 2000; Fulda et al., 2001). To this end, caspase-8 expression was found to be frequently inactivated by hypermethylation of regulatory sequences of the caspase-8 gene in a number of different tumor cells derived from neuroblastoma, malignant brain tumors, Ewing tumor, and small lung cell carcinoma both in vitro and also in vivo in primary tumor samples (Teitz et al., 2000; Fulda et al., 2001a,b). Importantly, restoration of caspase-8 expression by gene transfer or demethylation treatment sensitized resistant tumor cells for death-receptor- or drug-induced apoptosis (Fulda et al., 2001a,b). Conversely, enhanced transcription of caspase genes in response to cytotoxic treatment may increase expression levels. Thus, treatment with IFNγ resulted

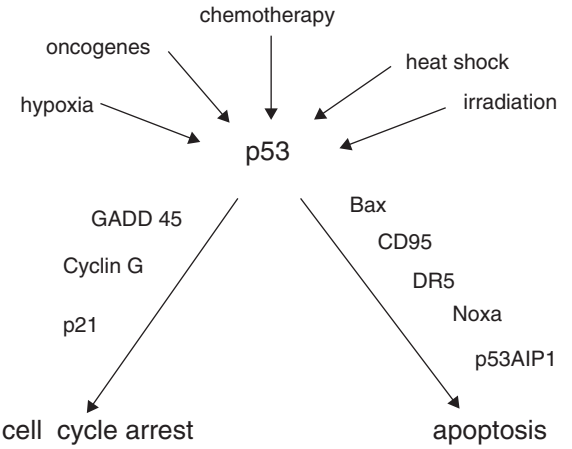


FIGURE 2. p53 and apoptosis. Various stress stimuli can activate the p53 protein, for example, chemotherapy, oncogenes, hypoxia, heat shock, or irradiation. p53 induces cell cycle arrest to ensure DNA repair or triggers apoptosis for elimination of damaged cells through the induction of various target genes. See the text for further details.

in enhanced expression of caspase proteins mediated by direct activation of STAT-1, a downstream transcription factor involved in IFNγ signaling (Fulda and Debatin, 2002). Moreover, transcriptional upregulation of caspase-3 or -8 was reported upon drug treatment independent of STAT1 (Micheau et al., 1999).

p53. As a sensor of cellular stress, p53 is activated by a variety of stimuli such as anticancer drugs, irradiation, hypoxia, oncogenes, or heat shock, leading to cell cycle arrest and/or apoptosis (Fig. 2; Vogelstein et al., 2000; Vousden, 2001). In response to cellular stress or DNA damage, p53 is phosphorylated at the N-terminus by two major kinases, ATM and Chk2, and also by ATR, casein kinase II, or the s46 kinase complex, which in turn results in stabilization of the p53 protein through inhibition of ubiquitin-mediated proteolysis (Vogelstein et al., 2000). Phosphorylation of the N-terminal residues alters the binding of p53 to Mdm2, while binding to the negative regulators JNK and Rb is controlled by caspase-mediated cleavage (Vogelstein et al., 2000). Also, phosphorylation of C-terminal residues of p53, acetylation, sumolation, or conformation changes can regulate p53 transcriptional activity (Vogelstein et al., 2000). The transcriptional activity of p53 is important for mediating its biological function and includes cell cycle regulatory genes such as p21, GADD45, reprimo, or 14-3-3, and apoptosis genes such as Bax, Noxa, p53AIP1, p53DINP1, CD95, TRAIL-R2, and PIG

(Vogelstein et al., 2000). In addition, transcription-indendent regulation of cell growth and apoptosis by p53 has also been described, for example, by promoting the translocation of CD95 from intracellular compartments such as the Golgi stores to the plasma membrane or by the direct effect of p53 on mitochondria (Vogelstein et al., 2000).

However, the relationship of p53, apoptosis, and the sensitivity of tumor cells to anticancer agents has been controversial. On the one hand, loss of p53 function has been shown to attenuate drug-induced apoptosis in vitro (Lowe, 1995). In addition, several clinical correlative studies and studies in mice showed an association between wt. p53 and chemosensitivity, indicating that p53 status may predict clinical response to chemotherapy (Wallace-Brodeur and Lowe, 1999). On the other hand, it has been proposed that p53 plays little or no role in the sensitivity of cancer cell to chemotherapy or radiation (Brown and Wouters, 1999). Although wt. p53 was found to predipose cells to die more rapidly by apoptosis as assessed by short-term assays, p53 status had no effect on clonogenic survival, indicating that p53 status may determine the threshold and kinetics of cell death rather than overall survival (Brown and Wouters, 1999). Also, p53 is not absolutely required for drug-induced cell death, since at sufficient doses virtually all anticancer agents induce apoptosis or other types of cell death independently of p53 (Brown and Wouters, 1999). Moreover, cells harboring wild-type p53 may fail to respond to cytotoxic treatment and those lacking functional p53 may even respond better (Brown and Wouters, 1999). Also, the contribution of p53 to apoptosis in response to cytotoxic therapies may depend on doses or tumor cell type and/or drug-specific patterns of modulation of chemosensitivity by p53.

## Antiapoptotic Signaling in Cancer Therapy

**Bcl-2 Proteins.** Bcl-2 family proteins play a pivotal role in the regulation of the mitochondrial pathway, since these proteins localize to intracellular membranes, in particular the mitochondrial membrane (Antonsson and Martinou, 2000). They comprise both antiapoptotic members, for example, Bcl-2, Bcl-XL, and Mcl-1, as well as pro-apoptotic molecules such as Bax, Bak, Bad, and BH3 domain-only molecules that link the death receptor pathway to the mitochondrial pathway (Bid, Bim, Puma, and Noxa) (Antonsson and Martinou, 2000; Oda et al., 2000; Cheng et al., 2001; Zhang et al., 2000). Upon apoptosis induction, pro-apoptotic Bcl-2 proteins with multidomains such as Bax translocate from the cytoplasm to the outer mitochondrial membrane, where they oligomerize to form a porelike structure, thereby promoting cytochrome c release (Cheng et al., 2001). This translocation to mitochondria can be triggered by Bcl-2 proteins that have a BH3 domain only (Cheng et al., 2001). BH3-domain-only proteins include Bid, which is activated by caspase-8-mediated cleavage; Bim, a microtubule-associated protein; or

Noxa and PUMA, two p53-induced proteins (Antonsson and Martinou, 2000). Bcl-2 or Bcl-$X_L$ exert their antiapoptotic function, at least in part, by sequestering BH3-domain-only proteins in stable mitochondrial complexes, thereby preventing activation and translocation of Bax or Bak to mitochondria (Antonsson and Martinou, 2000). In addition, Bcl-2 and Bcl-$X_L$ block apoptosis by preventing cytochrome c release through a direct effect on mitochondrial channels such as the voltage-dependent anion channel (VDAC) or the permeability transition pore complex (PTPC). Mutations or altered expression of pro- or antiapoptotic Bcl-2 family proteins can drastically alter drug response in experimental systems. In addition, several clinical correlative studies have supported the concept that high-level expression of antiapoptotic Bcl-2 proteins confers a clinically important chemoresistant phenotype on cancer cells, including AML, ALL, CLL, multiple myeloma, prostate carcinoma, malignant brain tumors, and neuroblastoma (Campos et al., 1993; Prokop et al., 2000). Likewise, reduced Bax levels have been associated with poor responses to chemotherapy and shorter overall survival in breast or colorectal carcinoma (Sturm et al., 2001; Bargou et al., 1995). Conversely, enhanced Bax levels correlated in several cell types with response to chemotherapy in vivo (Sturm et al., 2001).

Inhibitor of Apoptosis Proteins. "Inhibitor of apoptosis proteins" (IAPs) have been reported to directly inhibit active caspase-3 and -7 and to block caspase-9 activation (Deveraux and Reed, 1998; Holczik and Korneluk, 2001). In addition to regulation of apoptosis, IAP members such as survivin have been found to be involved in the regulation of mitosis (Altieri, 2001). The activity of IAPs is controlled at various levels, for example, by the transcription factor NFκB that has been reported to stimulate expression of cIAP1, cIAP, and XIAP (Deveraux and Reed, 1998). Inhibition of apoptosis by IAPs in response to cytotoxic therapy has been suggested by several experimental studies. XIAP, cIAP1, or cIAP2 suppressed apoptosis in vitro following treatment with cisplatin, cytarabine, TRAIL, or staurosporine, or after γ-irradiation (Datta et al., 2000; Altieri, 2001). Also, increased IAPs expression correlated with poor treatment response in myeloid leukemia cells and elevated survivin expression predicted adverse prognosis in several tumors, for example, neuroblastoma, AML, and colon, lung, and esophagus carcinoma (Tamm et al., 2000; Adida et al., 2000). IAPs are negatively regulated by caspase-mediated cleavage. In addition, Smac/Diablo and Omi, two proteins released from mitochondria upon apoptosis induction, neutralize IAPs through binding to IAPs, thereby displacing them from their caspase partners (Du et al., 2000; Suzuki et al., 2001; Martins, 2002). Likewise, XAF1 has been found to displace IAPs from bound caspases in the nucleus. Overexpression of Smac or Smac peptides sensitized even resistant tumor cells for apoptosis induction and strongly synergized with TRAIL to eradicate established tumors in an orthotopic mouse model of malignant

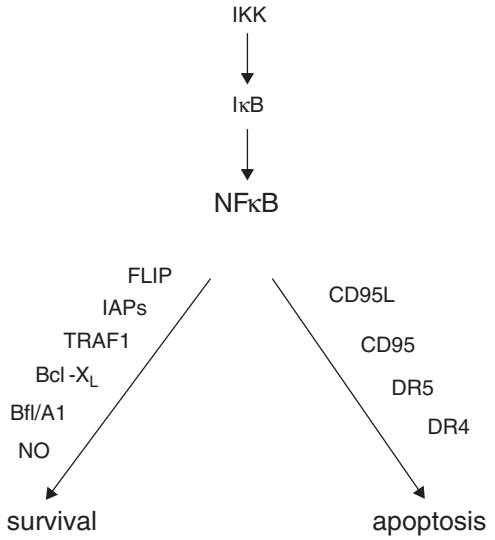


FIGURE 3. NFκB and apoptosis. NFκB is activated when its inhibitor IκB is degraded in response to increased activity of IKKs. NFκB can promote both apoptosis and survival through the induction of different sets of target genes.

glioma, indicating that Smac agonists represent novel promising cancer therapeutics (Fulda et al., 2002).

NFκB. The ability of the transcription factor NFκB to suppress apoptosis has been implied to confer resistance to cytotoxic therapies (Mayo and Baldwin, 2000). As outlined above, NFκB can already be constitutively active in certain tumor types such as pancreatic carcinoma (Mayo and Baldwin, 2000). In addition, NFκB activity is induced in response to a variety of stimuli, for example, in response to cellular stress and anticancer agents (Fig. 3) (Karin and Ben-Neriah, 2000). NFκB is composed of hetero- or homodimers of the NFκB/Rel family of proteins, which mediate protein dimerization, nuclear import, and specific DNA binding (Karin and Ben-Neriah, 2000). In most cell types, NFκB is sequestered in the cytoplasm by its interaction with IκB proteins and therefore remains inactive (Karin and Ben-Neriah, 2000). Upon stimulation, IκB becomes phosphorylated following activation of the IKK complex and is degraded via the proteasome, thereby releasing NFκB to translocate into the nucleus for transcription of target genes (Karin and Ben-Neriah, 2000). NFκB target genes include several anti-apoptotic proteins, for example, cIAP1, cIAP2, TRAF1, TRAF2, Bfl-1/A1, Bcl-$X_L$, and FLIP (Pahl, 1999; Barkett and Gilmore, 1999). Interestingly, promoter activation of certain pro-apoptotic factors such as CD95L, CD95, TRAIL-R1,

and TRAIL-R2 is also controlled by NFκB, consistent with reports that NFκB can promote apoptosis under certain circumstances (Pahl, 1999). The NFκB signaling pathway has been linked to death receptor signaling, since RIP that serves as an adaptor molecule for the TNFRI receptor to the NFκB pathway can be cleaved by caspases, thereby modulating the balance between pro- and apoptotic signals upon TNF receptor signaling (Herr and Debatin, 2001). Since certain types of anticancer treatments result in induction of NFκB transcriptional activity, inhibition of NFκB in parallel with chemotherapy strongly enhanced the cytotoxic effect of chemotherapy (Mayo and Baldwin, 2000). Thus, NFκB may play an important role in inducible chemoresistance, and inhibition of NFκB may serve as a potential new adjuvant approach to chemotherapy (Mayo and Baldwin, 2000).

PI3K/Akt. The PI3K/Akt pathway is a potent mediator of cell survival signals such as those delivered by growth factors or interactions with neighboring cells or with the extracellular matrix (Blume-Jensen and Hunter, 2001; Datta et al., 1999). Upon growth factor binding, transmembrane receptor tyrosine kinases undergo auto- and transphosphorylation, thereby recruiting PI3K to the plasma membrane where PI3K in turn recruits Akt via generation of phospholipids (Fig. 4) (Blume-Jensen and Hunter, 2001). Once activated, Akt regulates multiple signaling pathways involved in cell proliferation, apoptosis, glucose metabolism, or angiogenesis (Datta et al., 1999). The pro-survival function of Akt is mediated by phosphorylation of apoptosis signaling molecules such as Bad or caspases-9, or by inhibiting

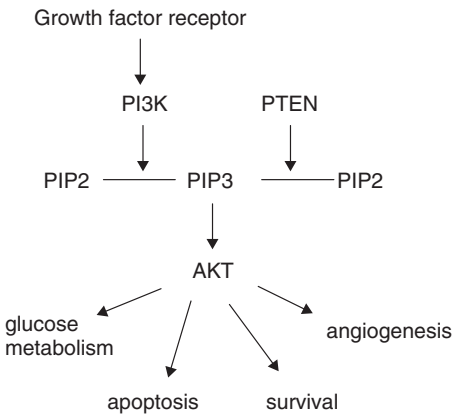


FIGURE 4. AKT and apoptosis. AKT activity is regulated in response to growth factor signaling by modulation of lipid metabolism. Activation of growth factor receptors or loss of the tumor suppressor gene PTEN results in AKT activation. AKT promotes survival by blocking apoptosis and by enhancing angiogenesis and glucose metabolism. See the text for further details.

cytochrome c release from mitochondria (Datta et al., 1999). The role of the PI3K/Akt pathway in treatment resistance has been suggested, since deregulated activation of Akt conferred resistance to apoptosis upon death receptor ligation or cytotoxic drug treatment (Datta et al., 1999). Thus, targeting the PI3K/Akt pathway, for example, by small-molecule inhibitors, may be useful to restore the sensitivity of tumor cells to cytotoxic therapies (Stein and Waterfield, 2000).

### Caspase-independent and Nonapoptotic Modes of Cell Death

Although a large body of data point to the essential role of caspase-dependent apoptosis in mediating tumor cell death upon cytotoxic therapy, this concept has also been challenged (Finkel, 1999). So far, a consistent link between the cells' ability to undergo apoptosis and their susceptibility to anticancer therapy could not be observed (Finkel, 1999). Thus, nonapoptotic modes of cell death, for example, necrosis or some forms of cell death that cannot be easily classified at present, have also been taken into consideration as a response to cytotoxic therapy (Leist and Jäättelä, 2001; Sperandio et al., 2001; Borner and Monney, 1999; Johnson, 2000). Also, delayed repression of tumors upon, for instance, irradiation has been interpreted as evidence against a predominant apoptotic mode of cell death, since apoptosis appears to be induced fairly rapidly in vitro and in vivo upon appropriate stimulation (Brown and Wouters, 1999). Although the signaling pathways and molecules involved in these alternative forms of cell death have not yet exactly been defined, noncaspase proteases such as calpains or cathepsins, Bax or Bax-like molecules, and AIF or endonuclease G may be involved (Johnson, 2000). The relative contribution of these different modes of cell death for chemoresponses in vitro and in vivo remains to be defined.

## Conclusion

Numerous studies over the last several years have indicated that cell death by apoptosis plays a crucial role in the surveillance of tumor formation and in anticancer therapies that primarily act by triggering apoptosis in tumor cells (Herr and Debatin, 2001; Kaufmann and Earnshaw, 2000; Johnstone et al., 2002). However, a few points remain to be addressed in future studies: First, most of the apoptosis signaling components have not been studied in clinical samples. Second, many experimental studies indicate that alterations in components of the apoptotic machinery have an impact on tumor formation and on sensitivity of tumor cells toward cytotoxic therapy; this premise remains to be tested in clinical settings. Moreover, the biology that determines the individual responses of different tumors to cytotoxic therapies

warrants further investigations to provide the basis for more specific therapeutic interventions. Finally, the concept that apoptosis represents the major mechanism by which tumor cells are eliminated by cytotoxic therapies may not universally apply, and caspase-independent apoptosis and nonapoptotic modes of cell death have also to be considered.

Nonetheless, studies on the role of apoptosis regulatory molecules in tumor development and in the response to anticancer therapies have provided substantial insights into cancer biology. Future studies on the role of apoptosis in individual tumors both in vitro and in vivo in tumor cells of patients under chemotherapy, for example, by DNA microarrays or proteomic studies, may provide the basis for "tailored" tumor therapy and may identify new targets for therapeutic interventions.

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CHAPTER

19

# The Role of Apoptosis in Myocardial Infarction and Heart Failure

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Apoptosis is a ubiquitous process that is critical for normal development, tissue homeostasis, and cell surveillance in all metazoan organisms. Given its importance in normal biology, it is not surprising that dysregulation of apoptosis—either too little or too much—can result in disease. This is illustrated by certain cancers in which inadequate apoptosis accounts, in large part, for the increase in tumor cells (McDonnell et al., 1989; Reed et al., 1988; Tsujimoto et al., 1985; Vaux et al., 1988). Conversely, an excess of apoptosis has been hypothesized to contribute to the pathogenesis of other disorders such as stroke, myocardial infarction, and heart failure (Thompson, 1995).

Cardiovascular disease is the most common cause of death in the developed world. Of the 2,400,000 annual deaths due to all causes in the United States, approximately 60%, or 1,400,000, are the result of cardiovascular disease (AHA, 2002). This figure far exceeds the total annual deaths from all cancers combined. The cardiovascular disorders primarily responsible for this extraordinary burden of mortality and disability are myocardial infarction, heart failure, and stroke. Although the pathogenesis of these syndromes is multifactorial, an increasing body of evidence suggests that

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apoptosis plays a significant role. In this chapter, we focus on the role of heart muscle cell apoptosis in myocardial infarction and heart failure and its potential therapeutic implications.

## Nature of the Problem: The Common Heart Syndromes

### Myocardial Infarction

Myocardial infarction is usually caused by the thrombotic occlusion of one or more of the coronary arteries, the blood vessels that carry oxygenated blood from the aorta to the myocardium or heart muscle (De Wood et al., 1980; Fallon, 1996). Thrombus (clot) formation is most often due to the rupture of a preexisting atherosclerotic plaque in the wall of the coronary artery (Falk et al., 1996). Typically, the plaque itself causes only a low-grade narrowing of the vessel. Following plaque rupture, however, a complex set of events triggers the recruitment of platelets and formation of thrombus that produces high-grade narrowing or complete occlusion of the coronary artery. This, in turn, causes myocardial ischemia, defined as decreased or absent blood flow to the myocardium. Ischemia results in the deprivation of oxygen, nutrients, and survival factors as well as the accumulation of waste products in the cells downstream of the occlusion, most notably heart muscle cells (cardiac myocytes) and cells lining the blood vessels (endothelial cells). Cell death ensues. Cardiac myocyte death during myocardial infarction has traditionally been thought to occur solely by necrosis. As will be discussed, this concept has been challenged by more recent work suggesting that a significant portion of cardiac myocyte death in human (Olivetti et al., 1996; Saraste et al., 1997) and rodent models of myocardial infarction (Bialik et al., 1997; Buerke et al., 1995; Fliss and Gattinger, 1996; Gottlieb et al., 1994; Kajstura et al., 1996; Palojoki et al., 2001) occurs by apoptosis.

Approximately 1,100,000 cases of myocardial infarction occur annually in the United States (AHA, 2002). Patients generally experience chest pressure that may spread into either arm, the neck, or jaw accompanied by shortness of breath and sweating. A cornerstone of acute therapy has been drugs that reduce the work which the heart muscle must carry out and, thereby, its need for oxygen. In addition, one of the most revolutionary developments in cardiology over the past 15 years, however, has been the recognition that the timely restoration of blood flow to the ischemic myocardium can reduce the size of a myocardial infarction and improve subsequent cardiac function and survival rates (1994; 1986). Accordingly, a second cornerstone of therapy is the reestablishment of blood flow through pharmacologic (thrombolytic agents) or mechanical (angioplasty or bypass surgery) means (Ryan et al., 1999). Although the *net* benefit of restoring blood flow to the ischemic

myocardium during myocardial infarction has been clearly demonstrated, reperfusion itself has been associated with some tissue damage primarily due to oxidative stress from the sudden reintroduction of oxygen-rich blood into a previously ischemic area (Bolli et al., 1989; Verma et al., 2002). Oxidative stress is a well-recognized apoptotic stimulus in diverse systems. Consistent with the importance of reperfusion-induced injury, most apoptosis during "ischemia-reperfusion" occurs in the reperfusion phase (Freude et al., 2000; Kang et al., 2000). Moreover, compared with continuous ischemia, ischemia-reperfusion appears to accelerate the onset of apoptosis but to reduce its overall magnitude (Fliss and Gattinger, 1996). Thus, reperfusion is a proven effective therapy for myocardial infarction, but it might be improved further by strategies to decrease reperfusion-related cell deaths.

## Heart Failure

The most general definition describes heart failure as a complex syndrome in which the heart is unable to pump sufficient blood to maintain homeostasis in the tissues in the body. A simpler definition that encompasses most cases is that heart failure is a weakening of the heart muscle. Patients experience fatigue, shortness of breath, and swelling of the feet and legs. (Despite the common meaning of "failure," the heart does not stop in heart failure unless the patient dies.) Approximately 4,900,000 Americans live with heart failure (AHA, 2002).

Heart failure can result from multiple disease states including high blood pressure, present or past myocardial infarctions, toxic insults to the myocardium (e.g., certain drugs, alcohol, viruses), problems with heart valves, and others. Prior to inducing heart failure, many of these stresses first elicit a compensatory response termed cardiac hypertrophy. Cardiac hypertrophy appears macroscopically as a thickening of the walls of the ventricles without much increase in the volumes of those chambers. During hypertrophy, cardiac myocytes, which are postmitotic terminally differentiated cells, grow by becoming larger in volume, rather than more numerous, and acquire increased intracellular protein with specific increases in the number of sarcomeres (contractile apparatus in striated muscle cells). In heart failure resulting from some causes (e.g., hypertension), hypertrophy occurs diffusely throughout the ventricles. In other situations (e.g., following myocardial infarction), hypertrophy is restricted to the remote, noninfarcted myocardium as the previously infarcted myocardium has usually become scar tissue. Although there is debate, hypertrophy probably functions as a compensatory mechanism to help the heart surmount stress. Over a period of months to years, however, this compensatory response fails and the ventricular chambers undergo enlargement, the walls change from thickened to thin, and contractile function deteriorates. This endpoint, termed dilated car-

diomyopathy, is the final common denominator of all forms of heart failure regardless of the initial inciting etiology.

The mechanisms that mediate the transition from compensated hypertrophy to dilated cardiomyopathy remain poorly understood. Although abnormalities of β-adrenergic signaling (Lefkowitz et al., 2000), $Ca^{++}$ handling and excitation-contraction coupling (Luo et al., 1994; Marks, 2002), cytoskeleton, (Chien, 1999), and myocardial energetics (Taegtmeyer, 2002) have been implicated in the pathogenesis of heart failure, none has emerged as the "main cause," perhaps because a single abnormality cannot explain this complex and chronic disorder. Recently, low, but abnormal, levels of cardiac myocyte apoptosis have been noted in rodent models of heart failure (Adams et al., 1998; Bisognano et al., 2000; Condorelli et al., 1999; Geng et al., 1999; Hirota et al., 1999; Li et al., 1997b; Liu et al., 1995; Palojoki et al., 2001; Sam et al., 2000; Sharov et al., 1996; Wencker et al., 2003; Xing et al., 2000; Zhang et al., 2000) and failing human hearts (Guerra et al., 1999; Olivetti et al., 1997; Saraste et al., 1999). We will consider these data below as well as studies suggesting that this low-level cell loss contributes to the pathogenesis of heart failure.

In summary, myocardial infarction and heart failure are two common and potentially lethal forms of heart disease. Myocardial infarction is an acute event that evolves over hours to 1 to 2 days. In contrast, heart failure is a chronic condition that usually develops over months to years. Chronic heart failure is often the result of prior myocardial infarctions, in which case it is the surviving, noninfarcted myocardium that "fails" over time.

## Apoptosis in Myocardial Infarction and Heart Failure: How much, when, and where?

### Myocardial Infarction

Cardiac myocyte apoptosis has been documented during human myocardial infarction (Olivetti et al., 1996; Saraste et al., 1997) as well as in rodent models of this syndrome involving intact animals (Bialik et al., 1997; Buerke et al., 1995; Fliss and Gattinger, 1996; Gottlieb et al., 1994; Kajstura et al., 1996; Palojoki et al., 2001), isolated hearts (Maulik et al., 1998; Chen et al., 2001a, 2001b), and isolated cardiac myocytes (Bialik et al., 1999; de Moissac et al., 2000; Kang et al., 2000; Malhotra and Brosius, 1999; Tanaka et al., 1994; von Harsdorf et al., 1999). Cardiac myocyte apoptosis has been demonstrated by various combinations of TUNEL, caspase activation, cytochrome c release, phosphatidylserine externalization, and electron microscopy.

During myocardial infarction (due to both continuous ischemia and ischemia-reperfusion), there is a large burst of cardiac myocyte apoptosis

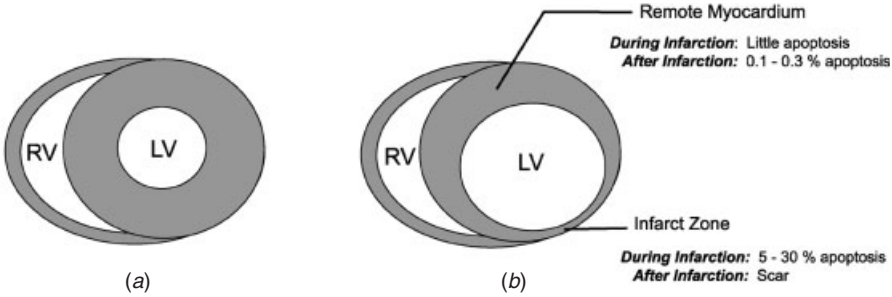


FIGURE 1. Apoptosis during and after myocardial infarction. Schematic representation of cross-section of normal heart (left) or heart following myocardial infarction (right). Right ventricle (RV) and left ventricle (LV) are indicated. There is a large burst of cardiac myocyte apoptosis in the infarct zone during myocardial infarction. With time, the infarct zone becomes hypocellular scar tissue. Weeks to months following infarction, a low, but abnormal, level of cardiac myocyte apoptosis occurs within the remote (noninfarcted) myocardium. For reference, the apoptotic rate in normal myocardium is 0.001%.

that can comprise 5 to 30% of the total number of cardiac myocytes within the ischemic zone (Bialik et al., 1997; Fliss and Gattinger, 1996; Kajstura et al., 1996; Fig. 1). This burst of apoptosis takes place over a relatively short period of time. It is first detectable after ~2 hours of continuous ischemia or ~1 hour of reperfusion following 45 minutes of ischemia (Fliss and Gattinger, 1996). According to one study that used a loss of membrane integrity assay to recognize necrosis in vivo, most of the cell death in the early hours of myocardial infarction is apoptosis, with necrosis peaking at 24 hours (Kajstura et al., 1996). As judged by DNA fragmentation, apoptosis is largely completed by 24 hours and not detectable after 48 hours (Bialik et al., 1997). In cultured cardiac myocytes stimulated by hydrogen peroxide, a surrogate for reactive oxygen species as with reperfusion, 14 hours were required for DNA fragmentation (Suzuki et al., 2001). Estimates are lacking, however, as to the precise time required for a cardiac myocyte to go from death stimulus to engulfment.

Much of the apoptosis in the ischemic brain is localized to the penumbra (Linnik et al., 1995), a border zone between ischemic and perfused tissue. In the heart, opinion is split as to the primary location of the acute burst of cell death during myocardial infarction. Most studies report that cardiac myocyte apoptosis occurs diffusely throughout the area at risk (Bialik et al., 1997; Fliss and Gattinger, 1996), whereas others note that apoptosis is of greater magnitude in the border zone (Palojoki et al., 2001; Saraste et al., 1997).

Following the high-intensity, short-duration burst of cardiac myocyte apoptosis in the ischemic zone during the acute infarction, low levels of apoptosis commence in the noninfarcted myocardium within 1 to 2 days and continue for months (Abbate et al., 2002; Baldi et al., 2002; Cheng et al., 1996; Olivetti et al., 1996; Palojoki et al., 2001; Sam et al., 2000; Saraste et al., 1997; Fig. 1). We will consider these remote regions in the discussion of heart failure.

In addition to cardiac myocytes, endothelial cells lining small coronary arteries also undergo apoptosis following ischemia-reperfusion (Scarabelli et al., 2001). Apoptosis in endothelial cells appears to take place even earlier than that in cardiac myocytes. In addition, there appears to be a radial pattern of cardiac myocyte apoptosis surrounding blood vessels with endothelial cell apoptosis. These observations suggest that endothelial cells and/or vessel damage may participate in the amplification and/or transmission of apoptotic stimuli to the myocytes.

## Heart Failure

In contrast to the high-frequency, short-duration pattern of cardiac myocyte apoptosis during myocardial infarction, the failing heart exhibits a very low, but still abnormal, frequency of apoptotic events that persist for weeks to months in rodents—and presumably months to years in humans. Although the magnitude of this cell death in human heart failure was initially overestimated probably due to technical issues (Narula et al., 1996), several subsequent studies have consistently demonstrated frequencies of cardiac myocyte apoptosis of 0.08 to 0.25%, while the baseline rate in normal hearts is ~0.001% (Guerra et al., 1999; Olivetti et al., 1997; Saraste et al., 1999; Fig. 1). Thus, although failing hearts exhibit very low frequencies of apoptosis, these frequencies are at least 80- to 250-fold above baseline.

In advanced heart failure of some causes, this cell death occurs diffusely throughout the ventricles. In the case of heart failure due to previous myocardial infarction, the distribution of cardiac myocytes apoptosis occurs primarily in the remote (noninfarcted) myocardium and border zones of the infarcts as the infarcts themselves have long since become scar tissue (Abbate et al., 2002; Baldi et al., 2002; Cheng et al., 1996; Olivetti et al., 1996; Palojoki et al., 2001; Sam et al., 2000; Saraste et al., 1997). Although the precise mechanisms that elicit cardiac myocyte apoptosis in the remote, noninfarcted myocardium are incompletely understood, it is likely that a combination of mechanical stresses due to the previous loss of contracting myocytes in the infarct and humoral factors induce this apoptosis. We will discuss below some of the stimuli that trigger cardiac myocyte apoptosis.

In summary, cardiac myocyte apoptosis during myocardial infarction is high-level, short-duration, and localized primarily in the central infarct zone. In contrast, apoptotic death of cardiac myocytes during heart failure is very

low-level, ongoing, and usually occurs diffusely except in the case of heart failure due to prior myocardial infarctions, in which instance it is localized to the remote, noninfarcted myocardium.

## Stimuli that Modulate Apoptosis

From the preceding discussion, it is evident that myocardial infarction and heart failure are complex pathophysiological processes. Accordingly, one would anticipate that numerous component stimuli play roles in the pathogenesis of these syndromes. Because of the inherent difficulties in manipulating individual stimuli in vivo, our knowledge in many cases is limited to whether a putative stimulus *can* induce cardiac myocyte apoptosis rather than whether that stimulus is actually responsible for cardiac myocyte apoptosis in a given pathological situation. Nevertheless, this information provides a beginning framework within which to place the signaling pathways that mediate cardiac myocyte apoptosis. Potential component stimuli for apoptosis during myocardial infarction and heart failure and their associated signaling pathways will be considered in this section, whereas the central death pathways with which they connect will be discussed in the following section.

Potential apoptotic stimuli during myocardial infarction include hypoxia, loss of nutrients, and loss of survival factors (during ischemia) and oxidative stress and calcium overload (during reperfusion). Hypoxia has been shown to induce apoptosis in cultured cardiac myocytes (de Moissac et al., 2000; Tanaka et al., 1994), although some data suggest that it is the concomitant acidosis that is the trigger (Webster et al., 1999). The mechanism of hypoxia-induced apoptosis in cardiac myocytes is not completely understood. Hypoxia increases levels of p53 in these cells. Moreover, overexpression of p53 in normoxic cells suffices to induce apoptosis. These observations suggest that hypoxia-induced apoptosis in cardiac myocytes may be p53-dependent (Long et al., 1997). Experiments using p53 knockout mice, however, demonstrate that p53 is not required for hypoxia-induced apoptosis in cultured cardiac myocytes and ischemia-induced apoptosis in vivo (Bialik et al., 1997; Webster et al., 1999). This lack of an absolute requirement, however, does not preclude a role for p53 in cardiac myocyte apoptosis.

Loss of nutrients, and metabolic stress in general, can also induce apoptosis in cardiac myocytes. An example is glucose deprivation, as during ischemia. In cultured cardiac myocytes, omission of glucose from the media induces little or no apoptosis. When combined with deprivation of serum factors (see below) and the addition of 2-deoxyglucose, a nonmetabolizable glucose analog that blocks glycolysis, however, more apoptosis results than with either stimulus alone (Bialik et al., 1999). Conversely, low to moderate

concentrations of glucose can suppress hypoxia-induced apoptosis in these cells (Malhotra and Brosius, 1999). In contrast, high concentrations of glucose appear to be pro-apoptotic in cardiac myocyte (Fiordaliso et al., 2001), in part due to activation of the local tissue renin-angiotensin system and p53 (Fiordaliso et al., 2000, 2001) and due to the production of reactive oxygen species (Shizukuda et al., 2002). The basis for the differences between the pro-apoptotic and antiapoptotic effects of glucose in these systems is not well understood. In addition to the differences in glucose concentrations, another possibility is differences in experimental systems. Most of the experiments showing the pro-apoptotic effect of glucose were carried out in adult cardiac myocytes, whereas experiments demonstrating a protective effect occurred in neonatal cells.

Serum contains a variety growth/survival factors whose access to the myocardium is severely impeded during ischemia. In cultured cardiac myocytes as in many other cell types, omission of serum from cell culture media induces apoptosis (Fujio et al., 1997; Sheng et al., 1997). Many serum factors function both to stimulate growth (cardiac myocyte hypertrophy in this case) and to inhibit apoptosis. One factor that is particularly well characterized with respect to cardiac myocytes is insulin-like growth factor-1 (IGF-1). In cultured cardiac myocytes, IGF-1 inhibits apoptosis induced by serum withdrawal (Fujio et al., 2000; Wang et al., 1998b) and by the cancer chemotherapeutic agent doxorubicin (Wang et al., 1998b), which incidentally can cause heart failure in part by inducing cardiac myocyte apoptosis. In intact animals, IGF-1 limits apoptosis and myocardial damage in the infarct zone during ischemia-reperfusion (Buerke et al., 1995) and during the chronic remodeling of the remote myocardium following permanent coronary occlusion (Li et al., 1997a). One mechanism of IGF-mediated protection in cardiac myocytes involves activation of the phosphatidylinositol 3-kinase (PI 3-kinase)–Akt axis (Fujio et al., 2000; Matsui et al., 1999). Other serum factors that block cardiac myocyte apoptosis include insulin (Aikawa et al., 2000), hepatocyte growth factor/scatter factor (Kitta et al., 2001, 2003; Nakamura et al., 2000), growth hormone (Gu et al., 2001), and basic fibroblast growth factor (Iwai-Kanai et al., 2002).

Oxidative stress is an important apoptotic stimulus during the reperfusion phase of ischemia-reperfusion. It is often modeled in cell culture by hypoxia followed by reoxygenation (Kang et al., 2000) or by the addition of hydrogen peroxide to the media (von Harsdorf et al., 1999), both of which efficiently induce cardiac myocyte apoptosis. Among many other cellular changes, oxidative stress stimulates increases in p53 and translocation of Bax and Bad to mitochondria (von Harsdorf et al., 1999), although the precise molecular mechanisms connecting the stimulus with these events is unknown.

Another component of ischemia-reperfusion is intracellular $Ca^{++}$ overload, which can result from intracellular acidosis, leading first to $Na^{+}/H^{+}$

exchange and then to $Na^+/Ca^{++}$ exchange across the cell membrane (Karmazyn, 1999). Although $Ca^{++}$ has long been suspected to be involved in some types of apoptosis, its role has not been precisely defined nor have the mechanisms by which it acts. In the heart, things are further complicated by the phasic 10-fold increases in intracellular $Ca^{++}$ concentration from 100 nM to 1 μM levels that occur with each heart beat, suggesting that if increases in $Ca^{++}$ do play a role in cardiac myocyte apoptosis, other mechanisms must also be present (Marks, 2003). These caveats notwithstanding, it is likely that increased intracellular calcium following ischemia-reperfusion contributes to mitochondrial calcium overload and dysfunction as well as the activation of calcium-activated enzymes such as calpain. In fact, calpain is activated by ischemia-reperfusion and can cleave Bid to produce a carboxy fragment capable of triggering cytochrome c release (Chen et al., 2001a, 2002). Further work is needed to determine the importance of this mechanism as well as to understand the full spectrum of apoptotic mechanisms that might result from increases in intracellular $Ca^{++}$.

Potential apoptotic stimuli during heart failure include mechanical stretch, adrenergic overstimulation, and certain cytokines and hormones. During hypertrophy and heart failure, pressures are elevated within the cardiac chambers. This tends to stretch the heart muscle. Mechanical stretch is a well-established stimulus for hypertrophic cardiac growth (Knoll et al., 2002). It can also induce cardiac myocyte apoptosis, however (Cheng et al., 1995). The mechanisms of stretch-induced apoptosis are likely to be complex because stretch activates numerous signaling pathways. Two mechanisms that may be involved are stretch-generated reactive oxy-gen species (Cheng et al., 1995) and angiotensin II, which is secreted by cardiac myocytes in response to stretch and then acts in an autocrine manner to stimulate hypertrophy (Sadoshima et al., 1993) and, as discussed below, death.

During heart failure, β-adrenergic tone is very high and serves to increase heart rate acutely and improve cardiac contractility, resulting in improved delivery of blood to the tissues of the body. This compensatory response is part of the ancient "fight-flight" response. It was perplexing, therefore, that medicines designed to augment β-adrenergic signaling, while improving cardiac function in the short term, were found to be detrimental when used chronically (Packer et al., 1991). Conversely, β-adrenergic receptor blockers were found to improve symptoms and mortality in heart failure (Packer et al., 1996). In retrospect, studies dating back more than 40 years have demonstrated cardiac toxicity from β-adrenergic stimulation (Rona, 1959). It is now known that β-adrenergic agents induce cardiac myocyte apoptosis (Communal et al., 1998; Shizukuda et al., 1998) Suggesting a possible mechanism for this toxicity. Specifically, $\beta_1$-adrenergic receptor activation induces apoptosis through a $G_s$-dependent mechanism, while $\beta_2$-adrenergic receptor activation is antiapoptotic, presumably through a $G_i$-dependent mechanism (Chesley et al., 2000; Communal et al., 1999; Geng

et al., 1999; Zaugg et al., 2000; Zhu et al., 2001). Although the mechanism by which $\beta_1$-adrenergic stimulation induces apoptosis was initially felt to occur through increases in cAMP and protein kinase A (PKA) activation (Communal et al., 1998), more recent studies suggest that this process may be independent of cAMP and PKA and involve $Ca^{++}$/calmodulin kinase II (Zhu et al., 2003).

Many patients with heart failure, as well as those with myocardial infarction, have elevated blood levels of the cytokine tumor necrosis factor-α (TNF) (Levine et al., 1990; Mann, 2003). In addition to various extracardiac sources, TNF is also produced by several cell types within the heart, including myocytes, to act as a paracrine/autocrine factor (Kapadia et al., 1995, 1997). When present at high levels for sustained periods, TNF can damage the heart in several ways. First, it can stimulate cardiac myocyte apoptosis (Krown et al., 1996; Sivasubramanian et al., 2001; D. L. Mann, personal communication). Second, independent of its apoptotic actions, TNF can elicit hypertrophy (Yokoyama et al., 1997), contractile dysfunction (Mann, 2003; Yokoyama et al., 1993), and degradation of the extracellular matrix (Sivasubramanian et al., 2001). Taken together, these cellular changes can promote dilated cardiomyopathy. In contrast to high, sustained levels of TNF, there is evidence that low concentrations for short periods of time are actually cardioprotective. The cardioprotection of TNF is most clearly illustrated by TNF receptor (TNFR) loss of function experiments. Although mice that lack either TNFR1 or TNFR2 exhibit normal-sized infarcts following ischemia-reperfusion, mice lacking both TNFR1 and TNFR2 exhibit infarcts that are *larger* than the wild type (Kurrelmeyer et al., 2000). Thus, in the setting of ischemia-reperfusion, the net effect of TNF is cardioprotective. The mechanism of TNF's cardioprotection may involve its transcriptional upregulation, through nuclear factor-κB (NF-κB), of several protective molecules, including manganese superoxide dismutase (MnSOD) (Wong and Goeddel, 1988), heat shock protein 72 (HSP72) (Nakano et al., 1996), inhibitor of apoptosis proteins 1 (cIAP1) and 2 (cIAP2) (Wang et al., 1998a), and c-FLIP (Micheau et al., 2001).

Interleukin-6 (IL-6) is another cytokine that is elevated in a variety of cardiac disease states. As in the case of TNF, IL-6 can elicit cardiac contractile dysfunction (Mann, 2003). IL-6 is a member of a family that also includes leukemia inhibitory factor (LIF), oncostatin M, ciliary neurotrophic factor, interleukin 11, and cardiotrophin-1 (CT-1). Interestingly, CT-1 and LIF are potent cardiac survival cytokines (Fujio et al., 1997; Sheng et al., 1996, 1997). Survival is mediated via the gp130 cell surface receptor through the mitogen-activated protein kinase (MAPK) pathway (Sheng et al., 1997), Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway leading to upregulation of *bcl-x* (Fujio et al., 1997), and the Akt pathway (Negoro et al., 2001). Mice with a ventricular-specific knockout of *gp130* are normal

at baseline but exhibit massive cardiac myocyte apoptosis, ventricular enlargement (heart failure), and organismal death in response to the imposition of a hemodynamic stress on the heart (Hirota et al., 1999). Thus, an important purpose of gp130-mediated signaling in cardiac myocytes may be to suppress stress-induced apoptosis to allow cardiac hypertrophy to take place in response to a hemodynamic stimulus.

Heart failure is also characterized by activation of the systemic renin-angiotensin system, resulting in arterial constriction and the retention of salt and water by the kidney (Francis et al., 1984). This "compensatory" mechanism may be good in a short-term emergency where maintenance of the blood pressure in the face of a failing heart is of paramount concern. In the long term, however, renin-angiotensin activation places a hemodynamic stress on the heart. The arterial constriction increases the resistance against which the heart must pump, and the retained fluid causes the cardiac chambers to be stretched. Given that stretch can elicit cardiac myocyte apoptosis (Cheng et al., 1995), it might be expected that these hemodynamic stresses would also activate this death program. In addition to these mechanical effects, angiotensin II can act directly on cardiac myocytes to induce apoptosis (Cigola et al., 1997; Kajstura et al., 1997). In fact, as described above, one of the mechanisms by which stretch elicits apoptosis may occur by stimulating the secretion of angiotensin II made by the local renin-angiotensin system in cardiac myocytes to act in an autocrine manner (Sadoshima et al., 1993). Angiotensin II-induced cardiac myocyte apoptosis requires the Type 1 angiotensin II receptor (Cigola et al., 1997; Kajstura et al., 1997) and appears to involve activation of protein kinase C (PKC) isoforms δ and ε (Kajstura et al., 1997) and increased intracellular $Ca^{++}$ (Cigola et al., 1997; Kajstura et al., 1997). Downstream events include increases in p53, resulting in activation of the genes encoding angiotensinogen (a precursor of angiotensin II), angiotensin II Type 1 receptor, and Bax, all of which are transcriptional targets of p53 (Leri et al., 1998; Pierzchalski et al., 1997). The production of additional angiotensinogen and angiotensin II Type 1 receptor results in a potential positive feed-forward loop. The role of p53 in this pathway is essential (Leri et al., 2000).

## Central Apoptotic Pathways in Cardiac Myocytes

Given the high degree of conservation of the central death pathways over evolution, it is not surprising that the basic features of these pathways in cardiac myocytes are similar to those in other cell types. There are, however, several striated muscle-enriched proteins that modify the regulation of these pathways in cardiac myocytes. We will first consider the evidence that the mitochondrial and death receptor pathways are operative and used in

cardiac myocytes, following which we will discuss how these pathways are modulated by cardiac myocyte-enriched proteins.

In the mitochondrial pathway (Green and Reed, 1998), various upstream signals, some conveyed by BH3-only proteins, impinge on the mitochondria and stimulate the release of apoptogenic molecules including cytochrome c, Smac (second mitochondrial activator of cytochrome c)/DIABLO (direct IAP-binding protein with low PI) (Du et al., 2000; Verhagen et al., 2000), AIF (apoptosis-inducing factor) (Susin et al., 1999), and Endo G (Li et al., 2001). Once in the cytoplasm, cytochrome c binds Apaf-1 (apoptotic protease-activating factor-1) along with dATP. This stimulates the oligomerization of Apaf-1 and the subsequent recruitment of pro-caspase-9 to form the apoptosome. Formation of this holoenzyme results in pro-caspase-9 activation, which is further augmented by pro-caspase-9 processing. Once activated, caspase-9 cleaves downstream effector pro-caspases.

The density of mitochondria in cardiac myocytes is one of the highest of all cell types. Not surprisingly, the mitochondrial pathway is activated by many apoptotic stimuli in these cells. This has been shown in cultured cardiac myocytes for hypoxia (de Moissac et al., 2000; Malhotra and Brosius, 1999), hypoxia-reoxygenation (Kang et al., 2000), serum/glucose withdrawal ± 2-deoxyglucose (Bialik et al., 1999), and reactive oxygen species (von Harsdorf et al., 1999). As with many other systems, cytochrome c translocation occurs in a caspase-independent manner (Bialik et al., 1999). Mitochondrial release of cytochrome c has also been observed in isolated, perfused rodent hearts (Chen et al., 2001a) and hearts of intact rodents (K. Mani, C.-F. Peng, P. Lee, unpublished observations) subjected to ischemia-reperfusion, and in infarcted human hearts (Narula et al., 1999). Moreover, Smac/DIABLO release has been observed in the hearts of intact rodents following ischemia-reperfusion (K. Mani, C.-F. Peng, R. N. Kitsis, unpublished observations). The importance of the mitochondrial pathway in disease pathogenesis is underscored by the 53 to 68% reductions in infarct size observed following ischemia-reperfusion in mice deficient in Bid (Peng et al., 2001), or with cardiac-specific overexpression of Bcl-2 (Brocheriou et al., 2000; Chen et al., 2001b) or a dominant negative mutant of pro-caspase-9 (Peng et al., 2001), as compared with wild-type mice (Fig. 2).

In the death receptor pathway (Ashkenazi and Dixit, 1998), soluble ligands (e.g., TNF) or cell membrane ligands (e.g., Fas ligand) bind to their cognate cell surface receptors. This induces a presumed conformational change in the receptor that causes its cytoplasmic tail to recruit an adaptor such as FADD (Fas-associated death domain protein) through interactions involving death domains in both molecules. FADD subsequently recruits pro-caspase-8 through homotypic interactions involving death effector domains. These proteins are some of the components of the DISC (death-inducing signaling complex). Once forced into close proximity, pro-caspase-8 autoactivates. Caspase-8 can then cleave and activate downstream

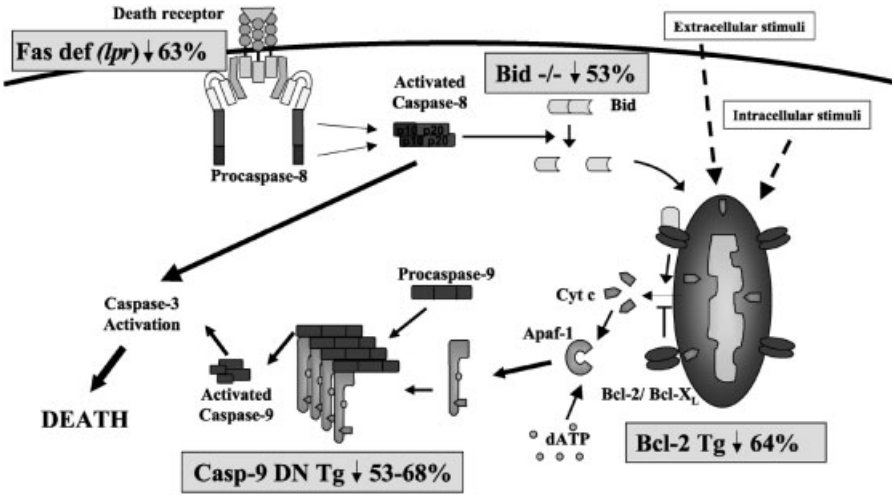


FIGURE 2. Multiple, independent mutations in apoptotic signaling molecules reduce myocardial infarct size in mouse models of ischemia-reperfusion. Def denotes deficient, Tg transgenic, DN dominant negative. Downward arrows and percentages indicate the percent reduction in myocardial infarct size of the given mutant relative to wild type.

effector caspases and/or activate the mitochondrial pathway by cleaving Bid.

Many investigators believed initially that the death receptor pathway was not important in cardiac myocytes. This was based on an experiment in which an activating Fas antibody was injected into a whole mouse (Ogasawara et al., 1993). The mouse died due to massive liver cell apoptosis. The hearts, however, appeared normal. Of course, there are several potential explanations for these observations in addition to the possibility that Fas signaling is unimportant in the heart. For example, liver cells and cardiac myocytes may have different sensitivities to Fas activation or require different amounts of time for pathology to be manifest. The ability of the Fas death pathway to be activated in cardiac myocytes was subsequently demonstrated in both cultured cells and intact animals using a recombinant adenovirus expressing Fas ligand (Lee et al., 2003). Induction of cardiac myocyte apoptosis by Fas ligand is a specific response because it is absent in cardiac myocytes in the *lpr* mice, a naturally occurring mutant that is deficient in Fas. Moreover, the importance of the Fas pathway for disease pathogenesis is emphasized by the 64% reduction in cardiac myocyte apoptosis and the 63% reductions in infarct size in *lpr* mice subjected to ischemia-reperfusion, as compared with wild-type mice (Lee et al., 2003; Figs. 2 and 3). Interestingly, cardiac myocyte apoptosis is reduced to a similar extent

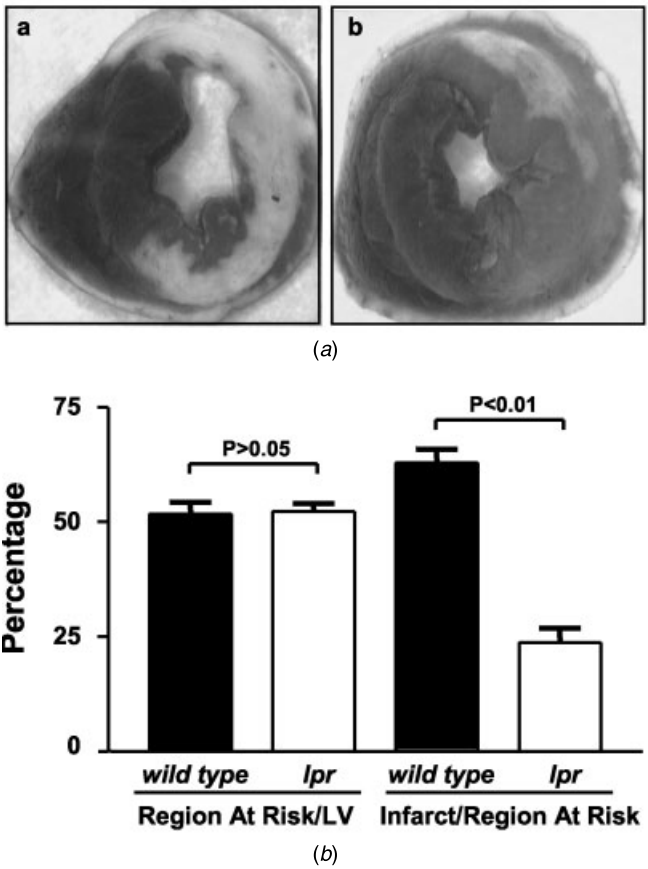


FIGURE 3. Example of reduction of myocardial infarct size during ischemia-reperfusion in Fas-deficient *lpr* mice. *Lpr* and wild-type mice were subjected to 30 minutes of left anterior descending coronary artery ligation followed by 24 hours of reperfusion. Following sacrifice, the region at risk (or ischemic zone) is identified by religating the coronary artery and infusing Evans blue dye. The absence of blue demarcates the region at risk. The heart is also incubated with 2,3,5-triphenyltetrazolium chloride (TTC), a measure of mitochondrial reductases, to assess viable tissue (red). Within the nonblue zone, the infarct is demarcated by the absence of red, which appears white. Panel A shows typical Evans blue/TTC staining of a wild type (a) and an *lpr* (b) heart. Note that, despite similar regions at risk (nonblue), the wild-type heart exhibits a much larger infarct (white) than the *lpr* heart. See color insert. Panel B shows the quantitative analysis for nine wild-type and eight *lpr* mice.

(56%) in *lpr* hearts studied as isolated, buffer-perfused (bloodless) preparations (Jeremias et al., 2000). This suggests that Fas deficiency in the heart, rather than blood cells, is responsible for the reduction in apoptosis. In addition, the isolated, perfused experiments demonstrated Fas ligand in the drainage from the heart following ischemia-reperfusion, showing that a source of Fas ligand is the heart itself, although the exact cell type has not been identified.

One connection between the death receptor and mitochondrial pathways is provided by Bid, a BH3-only pro-apoptotic Bcl-2 family member (Li et al., 1998; Luo et al., 1998). In many cell types, Bid is cleaved by caspase-8 that has been activated through death receptor signaling. The carboxy fragment of Bid containing the exposed BH3 domain then translocates to and inserts into the outer mitochondrial membrane. This stimulates Bak and Bax oligomerization and, through mechanisms yet to be elucidated, cytochrome c release (Wei et al., 2000, 2001). Bid undergoes cleavage and translocation to the mitochondria during ischemia-reperfusion in isolated, perfused hearts (Chen et al., 2001a; Scarabelli et al., 2002) and in intact animals (P. Lee and R. N. Kitsis, unpublished observations). As discussed previously, controversy remains concerning whether Bid cleavage during ischemia-reperfusion is mediated solely by caspase-8 (Scarabelli et al., 2002) or whether calpain also plays a role (Chen et al., 2001a). The importance of Bid in disease pathogenesis is highlighted by the 53% reduction in infarct size when mice deficient in Bid are subjected to ischemia-reperfusion, as compared to wild types (Peng et al., 2001; Fig. 2).

One cardiac myocyte-enriched protein that interacts directly with and modulates components of the central death machinery is ARC [apoptosis repressor with a CARD (caspase recruitment domain)]. Also referred to as CARD 2, ARC belongs to a multiprotein family of CARD-containing proteins (Bouchier-Hayes and Martin, 2002). Although some of these proteins are involved in the regulation of caspase activation and apoptosis (e.g., Apaf-1), some may have other primary roles (e.g., inflammation). ARC is expressed primarily in cardiac and skeletal myocytes and, to a limited extent, in the brain (Geertman et al., 1996; Koseki et al., 1998). It potently suppresses apoptosis induced by activators of both the death receptor and mitochondrial pathways (Ekhterae et al., 1999; Gustafsson et al., 2002; Koseki et al., 1998; Li et al., 2002; Neuss et al., 2001). ARC has been shown to interact with pro-caspases-8 and -2, and through its interaction with pro-caspase-8, to decrease death-receptor-induced caspase-8 activity (Koseki et al., 1998; Li et al., 2002). ARC also protects mitochondria from such stimuli as hypoxia and oxidative stress by inhibiting loss of mitochondrial membrane potential and cytochrome c release (Ekhterae et al., 1999; Neuss et al., 2001). The mechanisms that mediate these actions have not been elucidated. In short-term ischemia-reperfusion experiments, ARC has been shown to limit infarct size in isolated, perfused hearts (Gustafsson et al., 2002).

FLIP (Fas-associated death domain protein-like-interleukin-1 β-converting enzyme inhibitory protein) is another striated muscle-enriched apoptosis inhibitor (Irmler et al., 1997). FLIP is expressed as two isoforms. The short isoform consists of essentially two death effector domains, whereas the long form resembles pro-caspase-8 but lacks the active cysteine. FLIP inhibits death-receptor-induced apoptosis by binding both FADD and pro-caspase-8. FLIP levels are particularly high in cardiac muscle. During ischemia-reperfusion, however, FLIP abundance decreases markedly (Imanishi et al., 2000; Rasper et al., 1998). In other systems, apoptosis-induced decreases in FLIP abundance have been shown to be mediated by the ubiquitin-proteasome pathway (Fukazawa et al., 2001). Interestingly, FLIP plays an important role in heart development as mice deficient in this protein die in mid-gestation (embryonic day 10.5) due to maldevelopment of the myocardium. Somewhat paradoxically, a similar phenotype is observed in mice lacking FADD (Yeh et al., 1998) and pro-caspase-8 (Varfolomeev et al., 1998). Further investigation will be needed to determine whether these defects are cardiac myocyte autonomous and the common mechanisms involved in these knockouts.

BH3-only proteins such as Bid are thought to play the major activating role in the mitochondrial death pathway (Wei et al., 2001). Until recently, it was unclear which, if any, BH3-only proteins transmit pathological signals in heart disease. Two BH3-only proteins, Bnip3 (Kubasiak et al., 2002; Regula et al., 2002) and Nix/Bnip3L (Yussman et al., 2002), which are not cardiac-specific, have recently been noted to play important roles in hypoxia-induced and heart-failure-related cardiac myocyte apoptosis. Both of these proteins appear to be regulated at the level of transcription. Bnip3 mRNA and protein levels increase in response to hypoxia, and Bnip3 translocates to the mitochondria (Kubasiak et al., 2002; Regula et al., 2002), an event that may require the acidosis that accompanies hypoxia (Kubasiak et al., 2002). Cell death results. Bnip3 plays a critical role in hypoxia-induced cell death because cardiac myocytes are more resistant to hypoxia when Bnip3 levels are knocked down with antisense (Kubasiak et al., 2002) or when a Bnip3 mutant lacking a transmembrane domain necessary for mitochondrial memebrane insertion is expressed (Regula et al., 2002). Nix/Nip3L was found to be transcriptionally induced in a transgenic model of heart failure, resulting from overexpression of Gαq in the myocardium (Yussman et al., 2002). The rationale for this model is that Gq transduces the activation of several receptors (angiotensin II Type 1 receptor, α1-adrenergic receptor, endothelin receptor) that play roles in cardiac hypertrophy and failure (Dorn and Brown, 1999). These mice exhibit baseline cardiac failure (D'Angelo et al., 1997). In addition, during or after pregnancy, a percentage of the mice die due to fulminant heart failure accompanied by cardiac myocyte apoptosis (Adams et al., 1998). Nix/Bnip3 translocates to the mitochondria and stimulates cytochrome c release and cell death. Transgenic overexpression of

Nix/Bnip3 in the heart results in massive cardiac myocyte apoptosis and death of the animals. Conversely, transgenic overexpression of sNix, a splice variant of Nix/Bnip3 leading to a truncated cytosolic protein, decreases cardiac myocyte apoptosis, improves cardiac function, and attenuates the mortality during pregnancy of the Gαq transgenic mice (Yussman et al., 2002). Thus, Nix/Bnip3L appears to be a BH3-only protein that is important in the pathogenesis of heart failure. Interestingly, there is also evidence that Bnip3 is induced during heart failure as well (Regula et al., 2002).

## Cardiac Apoptosis as a Causal Component in Myocardial Infarction and Heart Failure

Although cardiac myocyte apoptosis occurs during myocardial infarction and heart failure, it need not necessarily play a role in the pathogenesis of these syndromes. In theory, this cell death could be merely an epiphenomenon or even serve some beneficial function. Accordingly, this section will examine the causal role, if any, of cardiac myocyte apoptosis in the pathogenesis of myocardial infarction or heart failure. The major question being asked here is: If apoptosis is removed, is the pathogenesis of these syndromes altered?

### Myocardial Infarction

Genetic disruption of various apoptotic signaling molecules has consistently demonstrated reductions of 53 to 68% in infarct size following ischemia-reperfusion in intact mice (Fig. 2). Thus, the *lpr* mouse (Fas-deficient) exhibits infarcts that are 63% smaller with 64% less cardiac myocyte apoptosis, as compared with wild types, following 30 minutes of ischemia and 24 hours of reperfusion (Lee et al., 2003; Fig. 3). Bid knockout mice show a 53% reduction in infarct size and improved cardiac function after being subjected to 45 minutes of ischemia and 24 hours of reperfusion (Peng et al., 2001). Cardiac-specific overexpression of Bcl-2 reduced infarct size by 64% and cardiac myocyte apoptosis by 61%, accompanied by improved cardiac function following 1 hour of ischemia and 24 hours of reperfusion (Brocheriou et al., 2000). Cardiac-specific overexpression of either of two independent dominant negative alleles of pro-caspase-9 reduced infarct size by 53 to 68%, markedly decreased cardiac myocyte apoptosis, and improved cardiac function following 45 minutes of ischemia and 24 hours of reperfusion (Peng et al., 2001). Since, as expected, cytochrome c release is not affected in the pro-caspase-9 dominant negative mice, some other caspase-dependent mechanism may be responsible for the marked reduction in infarct size in this model. Cardiac overexpression of Akt, which inactivates multiple apoptotic mechanisms, reduces infarct size by 50 to 64% and cardiac myocyte

apoptosis by 84%, and improves cardiac function following 30 minutes of ischemia and 24 hours of reperfusion (Matsui et al., 2001, 2002). Similar results were obtained in other studies with shorter periods of reperfusion (Chen et al., 2001b; Fujio et al., 2000; Miao et al., 2000). Thus, multiple independent mutations in apoptotic signaling molecules all yield the same answer: less apoptosis, smaller infarcts, and better cardiac performance than wild-type hearts subjected to the same insult.

To determine whether inhibition of cardiac myocyte apoptosis could be employed as a therapeutic modality, the effect of caspase inhibitors on myocardial ischemia-reperfusion has been assessed. Administration to rats of the peptide pseudosubstrate poly-caspase inhibitor zVAD-fmk starting prior to 30 minutes minutes of ischemia and continuing through 24 hours of reperfusion resulted in only a 22% reduction in infarct size despite a 73% reduction in cardiac myocyte apoptosis (Yaoita et al., 1998). Cardiac function improved mildly. Decreases of 31% in infarct size and 70% in cardiac myocyte apoptosis were observed in rabbits given YVAD-cmk starting prior to 30 minutes of ischemia and continuing through 3 hours of reperfusion (Holly et al., 1999). Another study in which YVAD-CHO or DEVD-CHO was initiated prior to 30 minutes of ischemia and continued through 6 hours of reperfusion in rats noted no reduction in infarct size, although a 40 to 50% decrease in cardiac myocyte apoptosis was observed (Okamura et al., 2000). Thus, despite inhibition of cardiac myocyte apoptosis, these standard caspase inhibitors resulted in mild to no reduction in infarct size and improvement in cardiac function following ischemia-reperfusion. In contrast, IDN-6734 is a dipeptide pseudosubstrate poly-caspase inhibitor that is at least two orders of magnitude more potent than zVAD-fmk. Infarct size was reduced by 52% and cardiac myocyte apoptosis by 64%, and cardiac function improved following 1 hour of ischemia and 7 days of reperfusion—even when dosing was initiated following 55 minutes of ischemia and continued for only 48 hours (Mani et al., 2002). Thus, delayed dosing of this potent caspase inhibitor resulted in sustained reductions in infarct size and improvements in cardiac function following ischemia-reperfusion.

## Heart Failure

Genetic manipulation of central apoptotic pathways has also been used to assess the role of cardiac myocyte apoptosis in the pathogenesis of heart failure. To test the sufficiency of cardiac myocyte apoptosis to cause heart failure, transgenic mice with cardiac-restricted expression of an inducible caspase-8 allele were created (Wencker et al., 2003). These mice develop heart failure and die over 2 to 6 months. Strikingly, an apoptotic frequency of only $23/10^5$ cardiac myocytes, which is 15-fold higher than controls but nevertheless a very small number, is sufficient to cause this lethal heart failure syndrome. Moreover, this apoptotic rate is actually 4- to 10-fold *lower* than

that observed in the hearts of patients with advanced heart failure. Thus, although there are limitations to cross-species comparisons, these data suggest that the small amount of cardiac myocyte apoptosis in failing human hearts may be important in pathogenesis as well. To test whether the low frequency of cardiac myocyte apoptosis in these transgenic mice was necessary for heart failure to develop, transgenics were treated with continuous long-term infusions of caspase inhibitors through osmotic minipumps. Caspase inhibition rescued most of the abnormalities in cardiac structure and function. Thus, in this model, a low level of cardiac myocyte apoptosis plays a critical role in the pathogenesis of heart failure. These data provide direct evidence that low levels of cardiac myocyte apoptosis can be a causal component of heart failure.

Several other studies have also examined the necessity of caspase activation and cardiac myocyte for heart failure in various models. Cardiac overexpression of Bcl-2 resulted in decreased cardiac myocyte apoptosis and improvement in cardiac dilation and function 6 weeks following myocardial in rabbits (Chatterjee et al., 2002). At 3 days postinfarct, there were similar degrees of cardiac dysfunction in the Bcl-2-treated and control groups, suggesting that infarct sizes were similar in the two groups. Thus, one interpretation of these data is that Bcl-2 ameliorated postinfarction myocardial remodeling in this study.

In a rabbit model in which heart failure is induced by rapid electrical cardiac pacing, the baculoviral caspase inhibitor p35 decreased cardiac myocyte apoptosis and improved cardiac function (Laugwitz et al., 2001). In addition to its effect on apoptosis, however, caspase inhibition also appeared to improve the structure and function of individual surviving cardiac myocytes. These data suggest that the positive effects of caspase inhibitors in heart failure may not be limited to inhibition of cell death. They may also improve the function of damaged, but living, cardiac myocytes. Observations suggesting that contractile proteins may be caspase substrates might provide one mechanism for this effect (Communal et al., 2002; Moretti et al., 2002).

Caspase inhibition was also tested in the previously described lethal peripartum cardiomyopathy exhibited by Gαq transgenic mice (Hayakawa et al., 2001). Administration of the dipeptide pseudosubstrate poly-caspase inhibitor IDN-1965 to pregnant Gαq mice markedly decreased cardiac myocyte apoptosis and moderately improved cardiac function. Remarkably, however, mortality was completely suppressed. Thus, although these effects may be due to suppression of apoptosis, functional improvements in individual cardiac myocytes, or both, inhibition of apoptotic signaling in small animal heart failure models appears to be beneficial.

The above studies provide hope that inhibition of apoptosis may provide a new therapeutic approach to myocardial infarction and heart failure. It is critical, however, that the reader recognize that large animal and human studies are essential to determine whether these observations in small

animal models will translate to the treatment of human disease. Even if inhibition of cardiac myocyte apoptosis is found to be beneficial in human myocardial infarction and heart failure, certain safety issues need to resolved. Foremost among these are the potential pro-carcinogenic effects of inhibiting apoptosis. Although this may not be a problem when inhibiting apoptosis for 24 to 48 hours as following myocardial infarction, it may be an issue with chronic therapy for heart failure. Even in this case, however, a weighing of the risks versus benefits may in the end determine whether a given therapy is appropriate for an individual patient. In addition, it may be possible to develop cell type-restricted approaches to apoptosis inhibition.

## Potential Therapies for Cardiac Myocyte Apoptosis

In this section, we will only comment on some of the potential therapeutic implications of the principles that have been discussed. Important considerations when thinking about antiapoptosis therapies include (1) premitochondrial versus postmitochondrial inhibition; (2) small-molecule versus gene therapy; and (3) cardiac-restricted versus systemic. For practical considerations, we will restrict our discussion to small molecules that are administered systemically.

β-adrenergic blockers are a class of medications with potentially antiapoptotic properties. They are in common usage as they have been shown to limit infarct size and decrease mortality in myocardial infarction (Freemantle et al., 1999; Hjalmarson et al., 1981) and to improve symptoms, cardiac function, and survival in heart failure (Lechat et al., 1998). The positive effects of β-blockers in these syndromes have been attributed to their decreases in myocardial oxygen consumption and/or antiarrhythmic effects. These agents also inhibit cardiac myocyte apoptosis in animal models of ischemia-reperfusion and heart failure (Asai et al., 1999; Sabbah et al., 2000; Yue et al., 1998). Given the previously described pro-apoptotic effects of β-adrenergic agonists in cell culture (Chesley et al., 2000; Communal et al., 1999; Zaugg et al., 2000; Zhu et al., 2001), it is possible that some of the positive effects of β-blockers are attributable to inhibition of apoptosis. Further studies will be required to test this possibility. If correct, however, one might predict from the cell culture studies that $\beta_1$ subtype-specific blockade would be more effective than nonspecific β-inhibition.

Inhibitors of the renin-angiotensin system, including angiotensin II-converting enzyme inhibitors and angiotensin II Type 1 receptor blockers, also have potential antiapoptotic effects. These drugs are widely used in human myocardial infarction, where they have favorable effects on post-myocardial infarction ventricular remodeling and mortality (Pfeffer, 1998), and in heart failure, where they improve symptoms and survival (Hunt et al., 2001). The beneficial effects of these agents are incompletely understood.

Although inhibition of the systemic renin-angiotensin system can produce favorable hemodynamic effects, beneficial effects in these disorders may also result from inhibition of the local renin-angiotensin system. Renin-angiotensin inhibition has been shown to decrease cardiac myocyte apoptosis in animal models of ischemia-reperfusion and heart failure (Goussev et al., 1998; Li et al., 1997b; Moudgil et al., 2001). In light of the direct anti-apoptotic effects of these agents (Cigola et al., 1997; Kajstura et al., 1997), it is again possible that inhibition of apoptosis may contribute to their effects in myocardial infarction and heart failure. As in the case of β-blockers, however, this speculation needs to be tested experimentally.

Sodium/hydrogen exchange blockers, such as cariporide, can reduce the intracellular $Ca^{++}$ overload associated with ischemia-reperfusion. These agents have been shown to reduce cardiac myocyte apoptosis and infarct size in animal models of ischemia-reperfusion (Chakrabarti et al., 1997; Linz et al., 1998). Human trials for ischemia-reperfusion have thus far been largely negative (Theroux et al., 2000), however, although subgroup analysis suggests that dosing may have been inadequate (Karmazyn, 2000). Further investigations are in progress.

IGF-1 and insulin have been shown to inhibit cardiac myocyte apoptosis and reduce infarct size in animal models of ischemia-reperfusion (Buerke et al., 1995; Gao et al., 2002; Jonassen et al., 2001; Yamamura et al., 2001). Decades before a role for cardiac myocyte apoptosis in heart disease was even contemplated, a cocktail of insulin-glucose-potassium was tested as an acute therapy for myocardial infarction. Although trials over the years have suggested a beneficial effect (Diaz et al., 1998), they have been inconclusive because of their small size or flawed design (Demots, 2001). A large trial is needed to provide a definitive answer to the usefulness of this therapy and whether any positive effects are attributable to inhibition of apoptosis or other actions (e.g., metabolic).

From a more general perspective, there are many growth/survival factors, some of which have already been shown to inhibit cardiac myocyte apoptosis in various paradigms. Some of these factors might be useful in the acute treatment of myocardial infarction or the long-term treatment of heart failure. For example, the gp130 receptor suppresses cardiac myocyte apoptosis induced by situations that require the heart to pump against an increased hemodynamic stimulus (e.g., hypertension) (Hirota et al., 1999). A gp130 ligand, such as CT-1, might provide a means to suppress cardiac myocyte apoptosis during myocardial infarction (Liao et al., 2002) or heart failure. In light of the growth-promoting effects of almost all these agents, potential undesirable effects such as cardiac hypertrophy and carcinogenesis need to be investigated.

Given the role of reactive oxygen species in apoptosis during ischemia-reperfusion and heart failure, the possibility is raised that pharmacological reduction of these species might be useful in the treatment of such condi-

tions. Although animal studies have demonstrated the clear benefits of this approach in ischemia-reperfusion (Dhalla et al., 2000), human studies have been negative (Flaherty et al., 1994; Lefer and Granger, 2000; Murohara et al., 1991). The reasons for this discordance are not well understood.

Mitochondrial $K^+$ ATP channels (Garlid and Paucek, 2001; Paucek et al., 1992), analogous to those on the plasma membrane, are thought to mediate a phenomenon known as preconditioning, in which repeated sublethal periods of myocardial ischemia limit the injury resulting from a subsequent more severe insult (Mei et al., 1996; Tomai et al., 1999). Although preconditioning has been demonstrated to reduce injury in animals and humans, the precise mechanism is unclear. Some drugs, such as diazoxide, open the mitochondrial $K^+$ ATP channel, inhibit loss of the mitochondrial membrane potential, reduce cardiac myocyte apoptosis, and limit infarct size in cell and animal models of ischemia-reperfusion (Akao et al., 2001, 2003; Takashi et al., 1999). Conversely, these effects are inhibited by 5-hydroxydecanoic acid, a mitochondrial $K^+$ ATP channel closer. The mechanistic relationship between the mitochondrial $K^+$ ATP channel and the mitochondrial membrane potential and apoptosis is not precisely understood. Moreover, in distinction to cell membrane $K^+$ ATP channels, the existence of mitochondrial $K^+$ ATP channels is supported mainly by pharmacological data; these channels have neither been isolated nor cloned. Thus, it remains possible that the positive effects of mitochondrial $K^+$ ATP channel openers are actually mediated by other actions of these drugs. Nevertheless, these drugs merit further investigation as treatments for ischemia-reperfusion.

From a conceptual perspective, the most specific antiapoptosis therapies would be those directed against targets in the central death machinery. There are many points within this machinery against which therapy could be directed. A potential advantage to intervening at a distal point (e.g., caspases) is that the redundancy of proximal pathways can be circumvented. A potential drawback to a distal target, however, is that it may lie beyond the point of no return for salvaging a cell. Thus far, the most widely tested small molecular inhibitors of the central death machinery have been caspase inhibitors. While the earliest such agents were tetrapeptide pseudosubstrates, later compounds are dipeptides or (nonpeptide) peptidomimetic compounds with improved cell permeability and pharmacological potency. As discussed in the previous section, experiments in rodents demonstrate that caspase inhibitors (1) reduce cardiac myocyte apoptosis and infarct size, resulting in sustained improvements in cardiac function following ischemia-reperfusion, and (2) reduce cardiac myocyte apoptosis and improve cardiac function and survival during heart failure. Given these promising results, it is important that these agents be tested in large animal models prior to possible clinical trials. Important endpoints will include not only efficacy but also safety, especially with respect to cancer, when long-term dosing is contemplated.

In the future, it is likely that additional small molecules will be identified that can inhibit more upstream events in the central death pathways (e.g., BH3-only proteins). Experiments will be required to determine how these approaches compare to caspase inhibition and whether synergism results from combining pre- and postmitochondrial therapies.

## Conclusion

In this chapter, we have reviewed mechanisms that mediate cardiac myocyte apoptosis and the increasing evidence that this cell death plays a critical role in the pathogenesis of myocardial infarction and heart failure. These data suggest that inhibition of cardiac myocyte apoptosis may provide a novel therapeutic modality for these common heart syndromes. Further studies in large animals will be critical before proceeding to human trials.

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