Apoptosis in Toxicology

Edited by

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Foreword

For many years it was thought that chemical-induced cell injury and death occurred primarily by necrosis. Now, however, it is recognized that cell death may also be the result of another major mechanism, namely apoptosis. It is realized that apoptosis is of fundamental importance in many areas of cell biology, including pathology, immunology, developmental biology, carcinogenesis and, more recently, pharmacology and toxicology.

The early pioneering work of Andrew Wyllie and his colleagues described the importance of apoptosis in various tumours and in certain forms of chemical-induced injury. Subsequent studies by Horvitz and colleagues, in understanding programmed cell death in the nematode *Caenorhabditis elegans,* recognized the importance of three genes, namely CED-3, CED-4 and CED-9, as being critically important for developmental cell death. During normal development, 131 cells of the 1090 cells generated die by apoptosis. Two genes, CED-3 and CED-4, are vital for cell death in *C. elegans,* while the CED-9 gene antagonizes their function and prevents cell death. Over the past few years, the mammalian homologues of these genes have been identified: the BCL2 family members (CED-9), the caspases (CED-3) and Apaf-1 (CED-4). The identification of these mammalian homologues has led to a rapid increase in our knowledge of the fundamental mechanism(s) of apoptosis, so enabling us to begin to define its role in many different toxicological scenarios.

The importance of apoptosis in toxicology has long been underestimated, largely because of the difficulty in identifying apoptotic cells in the intact organism, due to their rapid phagocytosis and disposal. New methods providing better identification and quantitation of apoptotic cells have aided in the recognition of the importance of apoptosis in various areas of toxicology. While most of the methods are applicable largely to *in vitro* systems, some newer methods are being developed to aid in the early recognition of apoptotic cells *in vivo* in tissues exposed to toxicants.

The present book deals with basic mechanisms of apoptosis and their relevance to toxicology, and several chapters highlight the importance of apoptosis in various tissues in response to toxic agents. This volume is an important contribution to understanding the role of apoptosis in toxicology and will be of particular value to those working on fundamental mechanisms of toxicity and cell death.

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CHAPTER 1

Apoptosis: Basic Mechanisms and Relevance to Toxicology

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1.1 Introduction
1.2 Morphology of apoptosis
1.3 Biochemical events during apoptosis induced by a toxicant
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1.1 INTRODUCTION

The death of a cell was thought for many years to be an uncontrolled, degenerative and catastrophic failure of homeostasis in response to cellular injury and was thus of little scientific interest. However, the discovery over the past two decades that all cells within multicellular organisms have the ability to activate a cell death programme termed apoptosis has challenged this idea and resulted in a re-evaluation of all aspects of biology including immunology, development, carcinogenesis, and more recently, toxicology. Toxicologists have known for many years that cells can be killed by a variety of toxicants and pathological conditions. In general, when the toxicant is at high enough concentration to cause gross cellular injury or a major perturbation in the cellular environment the cell dies by necrosis or 'cell murder'. However, it is more common for the affected cells to be deleted by apoptosis or 'cell suicide'. It is now believed that apoptosis is the major form of patho-physiological cell death and that necrosis is much rarer, occurring only in circumstances of gross cell injury (reviewed in Raffray and Cohen, 1997).

Apoptosis is desirable to the organism as a whole since it provides a mechanism for the disposal of cells damaged by mutagenic chemicals or irradiation without perturbing the homeostatic balance of its environment. In addition to a role during a cellular response to toxicants, apoptosis occurs throughout development and is vitally important in the immune system (Jacobson *et al.,* 1997). Examples include the removal of interdigital cells from a solid limb paddle during digit formation (Hammar and Mottet, 1971) and deletion of potentially lethal self-reactive B- and T-lymphocytes in the immune system (MacDonald and Lees, 1990). Moreover, apoptosis is of major importance in the pathogenesis of several diseases (Thompson, 1995) such as cancer (Dive and Wyllie, 1992, Lee and Bernstein, 1995, Reed, 1995), AIDS (Gougeon and Montagnier, 1993) and neurological disorders such as Alzheimer's and Parkinson's disease (Carson and Ribeiro, 1993, Bredesen, 1995). Therefore, a basic understanding of the molecular mechanisms that regulate apoptosis will have widespread implications for the prevention and treatment of these diseases. Apoptosis is of major interest to molecular toxicologists since it is central to the action of many toxicants and, as such, is important in the study of toxicological potential.

1.2 MORPHOLOGY OF APOPTOSIS

The execution phase of apoptosis has striking morphological characteristics (Alison and Sarraf, 1995, Raffray and Cohen, 1997, Zamzami *et al.,* 1998) although, at present, the latent phases of apoptosis are undetectable. The morphology of the end stages of apoptosis is remarkably similar across a wide variety of tissues; an apoptotic body from the liver may be indistinguishable from an apoptotic body from the crypt of the small intestine (Alison and Sarraf, 1995). These morphological changes are described in detail and illustrated in

Chapter 10. Briefly, they include loss of cell contact with neighbouring viable cells, chromatin condensation to form dense compact masses and breakdown of the cytoskeletal scaffolding. The cell surface membrane, which is already contracted, begins to ruffle and bleb. This is followed by breakdown of the cell into membrane-bound fragments or 'apoptotic bodies'. In contrast to necrosis, the plasma membrane and cytoplasmic organelles of the apoptotic cell remain intact. The apoptotic cell is swiftly recognized and engulfed then degraded by professional or amateur phagocytes (macrophages and neighbouring cells, respectively) (Savill *et al.,* 1993). *In vitro,* in the absence of phagocytosis, a secondary non-specific degeneration occurs which results in the uptake of vital dyes such as trypan blue and is commonly mistaken for necrosis. This process is often referred to as secondary necrosis (see Chapter 10). Importantly, the whole apoptotic process occurs without the development of an inflammatory response and thus facilitates the deletion of cells with minimal disruption to the surrounding cellular environment and tissue architecture.

1.3 BIOCHEMICAL EVENTS DURING APOPTOSIS INDUCED BY A TOXICANT

As with proliferation and differentiation, apoptosis has conserved, regulated stages. There also appear to be as yet incompletely defined biochemical checkpoints in the pathway to apoptosis where the cellular decision to live or die is made (Figure 1.1). The biochemical stages of apoptosis explained in this chapter are conserved throughout most of the tissues discussed in this book. There are events which occur prior to cellular commitment to apoptosis and others which are post commitment events. However, the events which actually commit a cell to apoptosis remain elusive. The idea of commitment, however, is an important one especially when considering sites for therapeutic intervention and molecular targeting in diseases where normal control of apoptosis has been lost or when considering antidotes to be applied after exposure of cells to a toxicant.

The biochemical stages of apoptosis induced by a toxicant are illustrated in Figure 1.1 and can generally be summarized as:

(1) the *imposition* of damage by the toxicant
(2) *sensing* the damage imposed by the toxicant
(3) *coupling* the damage to the engagement of apoptosis and
(4) *execution* of the cell and disposal of the corpse.

Each of these stages will be described in turn below. In addition to apoptosis induced by a toxic substance, apoptosis can be induced by the absence of necessary survival signals (see section 1.4.1) or via transmission of a lethal signal via activation of members of a family of death receptors including Fas (CD95/APO-1) (see section 1.3.2).

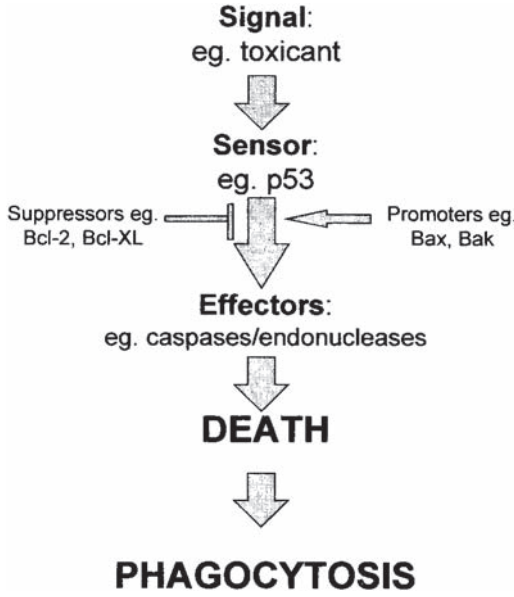

Figure 1.1 The stages of apoptosis: signal, sensor and execution.

1.3.1 Imposition of damage by a toxicant

Apoptosis can be induced by a plethora of toxicants which have different mechanisms of action including agents which damage DNA and nongenotoxic agents.

1.3.2 Sensing the damage imposed by the toxicant

How do cells monitor toxicant-induced damage? This important question has yet to be fully answered particularly for nongenotoxic agents. However, more is known about responses to DNA damage. Since DNA contains all the 'instructions' for the function of the cell and the future of further cell populations, its integrity must be of the highest order. Any mutations, adducts or loss of integrity of the DNA may lead to the loss of function of the cell or the development of a neoplastic potential. Therefore, a tissue disposes of cells with irreparable DNA lesions or a mutagenic potential by apoptosis. DNA damage may be on either one or both strands of the DNA, and may be caused by many agents such as ionizing radiation or the DNA alkylating nitrogen mustards (by direct DNA damage), or as a result of agents such as the topoisomerase II inhibitor etoposide (by damaging DNA indirectly). There are many candidate proteins which are known to respond to DNA damage such as poly (ADP-ribose) polymerase (PARP), DNA protein kinase (DNA PK), ATM, the c-abl tyrosine kinase and the tumour suppressor p53, but apart perhaps from p53, their roles in sensing DNA damage and coupling it to repair, arrest or apoptosis have yet to be well characterized.

1.3.2.1 The tumour suppressor gene p53

The p53 gene encodes a nuclear phosphoprotein which acts as a transcription factor and a 'sensor' of DNA damage (Kastan, 1996). Normally, p53 protein is kept at a low basal cellular concentration due to its relatively short half-life. In addition, p53 probably exists in a latent form, inactive for transcription (Levine, 1997). Exposure of cells to DNA-damaging agents leads to an increase in p53 protein levels which is thought to result from a combination of both an increase in p53 protein half-life by post-translational stabilization and an increase in p53 protein translation (Levine, 1997). This increase in the levels of p53 in the cell results in the activation of p53 as a transcription factor. The activation of wild-type p53 leads to either growth arrest in G1 (Kastan *et al.,* 1991) and/or the induction of apoptosis (Yonish-Rouach *et al.,* 1991). Therefore, p53 functions as a cell cycle checkpoint between G1 and S-phase preventing the progression of damaged or potentially tumourigenic cells. Growth arrest allows time to repair the damage before replication, or if the damage is too great to commit to apoptosis (Hale *et al.,* 1996). The function of p53 as a tumour suppressor is exemplified by p53-null mice which develop normally but are prone to a variety of spontaneous tumours by 6 months of age (Donehower *et al.,* 1992). The coupling between DNA damage, p53 induction and apoptosis is cell type specific both *in vivo* and *in vitro*. This is demonstrated by studies of the effects of whole body irradiation on the individual tissues of the mouse (Midgley *et al.,* 1995, MacCallum *et al.,* 1996); although the whole body received a DNA damaging dose of radiation, apoptosis was observed in the small intestine, thymus and spleen, but not the lung, muscle or liver. Lack of apoptosis in the thymus and spleen following irradiation of p53-null mice confirms the requirement for p53 (Komarova *et al.,* 1997). Apoptosis following DNA damage was initially believed to be completely p53 dependent in small intestinal epithelia (Merritt *et al.,* 1994), but further investigations using p53-null mice demonstrated a delayed initiation of apoptosis compared to wild-type mice, indicating the presence of a p53-independent mechanism (Merritt *et al.,* 1997 and see below).

In most experimental systems, p53-dependent apoptosis is dependent on p53-mediated transcription, although this remains controversial (reviewed in Levine, 1997). Proteins which have been shown to be regulated by p53 include members of the Bcl-2 family (Miyashita *et al.,* 1994, Selvakumaran *et al.,* 1994); it has been shown to up-regulate the synthesis of the pro-apoptotic protein, Bax, and to down-regulate levels of the antiapoptotic member Bcl-2 (Miyashita *et al.,* 1994). Since the intracellular balance of the proapoptotic Bcl-2 family members to the anti-apoptotic members has been demonstrated to regulate apoptosis in a variety of cell types, p53 would be predicted to induce cell death by tipping the Bcl-2 family rheostat towards apoptosis (Korsmeyer *et al.,* 1993).

Another gene which has been recently associated with DNA damage-induced, p53-dependent apoptosis is *fas* (CD95/APO-1). Fas is a member of the tumour necrosis factor receptor (TNF-R) superfamily (Cleveland and Ihle, 1995). Upon binding of its ligand

(FasL) Fas trimerizes and an intracellular receptor bound complex is assembled termed a death-inducing signalling complex (DISC) which in turn activates a caspase cascade (Chapter 2) leading to the death of the cell (Kischkel *et al.,* 1995). Increased transcriptional activity of p53 appeared to play a role in up-regulation of Fas in doxorubicin-treated hepatoblastoma cells (Muller *et al.,* 1997). In addition, there are temperature sensitive mutants of p53 which can up-regulate Fas (Owen-Schaub *et al.,* 1995). More recently, the TRAIL (TNF-related apoptosis inducing ligand) receptor-5 (also called KILLER/DR5), another member of the TNFR superfamily, has been shown to be induced in a p53-dependent manner following exposure of a variety of wild-type p53 containing cells to DNA damaging agents (Wu *et al.,* 1997).

p53 also transcriptionally up-regulates p21WAF1/CIP1 (El-Diery *et al.,* 1993) which acts as a potent inhibitor of cyclin dependent kinases (CDKs) causing cell cycle arrest at the GI/S border (El-Diery *et al.,* 1994). p21WAF1/CIP1 itself may also play a role in the regulation of apoptosis although this may be via a p53 independent mechanisms and is also cell context specific (El-Diery *et al.,* 1994, Polyak *et al.,* 1996). p53 also results in the up-regulation of the MDM2 protein (Hainaut, 1995, Hale *et al.,* 1996) which acts in a regulatory negative feedback loop by binding to p53 and blocking its transactivation (Chen *et al.,* 1994). This would lead to down-regulation of p53-dependent genes, such as those involved in apoptosis.

After DNA damage has caused a p53-mediated G1 arrest, DNA damaged cell can progress down three possible pathways: remain in a G1 arrest pending repair, repair the DNA and return to the cell cycle or die via apoptosis. The decision to undergo cell cycle arrest, repair or apoptosis is dependent upon cell type, the level of DNA damage received and the trophic environment. For instance, p53 is present in the liver but does not appear to accumulate following DNA damage induced by irradiation (Midgley *et al.,* 1995) or following administration of the cytotoxic drug etoposide (Gill *et al.,* 1998). Also, if mice are irradiated, the p53 protein is strongly expressed in the small intestinal crypts but not in the large bowel crypts (Merritt *et al.,* 1994). This may explain why tumours are more common in humans in the large bowel compared with the small bowel (Alison and Sarraf, 1995).

The role of p53 may be of major importance to toxicologists with reference to risk assessment. For instance, at high doses of a genotoxic chemical, cells may engage the apoptotic pathway. At lower doses, below the threshold level, cells may growth arrest, survive and thereby increase its chance of mutation. However the existence of p53 independent pathways linking DNA damage to apoptosis must also be considered.

1.3.2.2 p53 independent mechanisms for sensing DNA damage

p53 independent mechanisms to 'sense' DNA damage certainly exist. For example, in mice null for p53, apoptosis occurred following irradiation, but at a later time-point to that observed in wild-type mice (Merritt *et al.,* 1997). One molecule implicated in

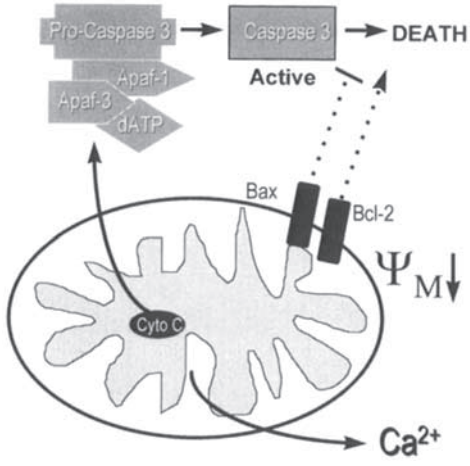

Figure 1.2 The principal molecular regulators of apoptosis.

p5 3-independent DNA damage response pathways is interferon regulatory factor 1 (IRF-1) (Nozaki *et al.,* 1997) since G1 arrest and apoptosis were lost in DNA-damaged IRF-1 null fibroblasts and in IRF-1 null spleen-derived mature T-lymphocytes, respectively. DNA damaging agents activate the nuclear tyrosine kinase c-Abl, considered to be a *sensor* of DNA damage involved in coupling DNA damage to apoptosis (Kharbanda *et al.,* 1995). Cells which are null for c-Abl are more resistant to DNA damage-induced apoptosis than their wild-type counterparts (Yuan *et al.,* 1997). c-Abl binds to p53 at least *in vitro* but appears to have a pro-apoptotic function in the presence or absence of p53.

1.3.3 Coupling cellular damage to the engagement of apoptosis

After cellular damage has been *sensed,* this information has to be processed and coupled to a cellular response to the damage, and the decision to survive or to die must be taken. There is very little known about how these cellular processes might occur but the molecules and mechanisms implicated are discussed below.

1.3.3.1 Mitochondrial events

Mitochondria play a central role in mediating apoptosis and appear to be recipients of damage-induced signals (reviewed by Kroemer *et al.,* 1995). There is a loss of electrical potential across the inner mitochondrial membrane (Ψm) early in the apoptosis pathway (Figure 1.2) which is termed the mitochondrial permeability transition (PT) (Kroemer *et al.,* 1995, Zamzami *et al.,* 1998). Cytochrome c release from mitochondria has also been shown to be a central event in the regulation of apoptosis (Liu *et al.,* 1996, Kluck *et al.,*

1997, Yang *et al.,* 1997). Once released from the mitochondria, cytochrome c (also termed Apaf-2, apoptosis protease activating factor-2) binds to an adaptor protein Apaf-1. This is the mammalian homolog of the nematode death gene *ced-4* which acts upstream of the worm caspase *ced-3* and downstream of the worm homolog of Bcl-2, *ced-9* (see Chapter 2, section 2.2.2). Apaf-1 has also been demonstrated to bind to dATP and caspase 9 (Apaf-3) (Vaux, 1997, Zou *et al.,* 1997). This complex of Apaf-1,-2,-3 and dATP is thought to trigger the cleavage of pro-caspase 3 which in turn initiates a caspase cascade, the executionery machinery of apoptotic cell death (discussed in Chapter 2, section 2.2.2). Exactly how cytochrome c is released from mitochondria into the cytosol is unclear but one hypothesis is that the outer mitochondrial membrane ruptures permitting the release of cytochrome c from the inter-membrane space (Zamzami *et al.,* 1998). The rupture of the outer mitochondrial membrane is believed to occur due to a PT-mediated influx of ions and water into the mitochondrial matrix. Since the inner mitochondrial membrane has a larger surface area than the outer membrane, the former could expand until it physically ruptures the latter (reviewed in Zamzami *et al.,* 1998). The relationship between PT and the release of apoptogenic substances such as cytochrome c and apoptosis inducing factor (AIF) from the mitochondria into the cytosol is currently unclear. The PT pore is not large enough to allow passage of cytochrome c. The release of cytochrome c from mitochondria can be regulated by members of the Bcl-2 family (1.1.3.2 and Chapter 2, section 2.2.2). A perplexing question remains unanswered: how does damage induced by a diverse array of toxicants with widely different cellular targets result in the induction of the mitochondrial events described above?

1.3.3.2 Intracellular calcium

One signalling molecule which could conceivably link diverse types of cellular damage to the mitochondrial events involved in mediating apoptosis is intracellular calcium, $[Ca^{2+}]_i$. Early studies suggested that alterations in $[Ca^{2+}]_i$ homeostasis were frequently observed in cells undergoing apoptosis (McConkey and Orrenius, 1997). However, it was never clear whether this was a cause or consequence of the apoptotic process. A role for $[Ca^{2+}]_i$ in regulating apoptosis was supported by the observation that $[Ca^{2+}]_i$ buffering agents, Ca^{2+} chelators and inhibitors of the endoplasmic reticular (ER) Ca^{2+}-ATPase can inhibit caspase activation, DNA fragmentation, and cell death (McConkey and Orrenius, 1997). Conversely, calcium ionophores, which increase $[Ca^{2+}]_i$ trigger apoptosis in several cell types (McConkey and Orrenius, 1997). Further support for the involvement of Ca^{2+} influx in the triggering of apoptosis comes from studies with specific Ca^{2+} channel blockers, which abrogate apoptosis in the regressing prostate following testosterone withdrawal and in pancreatic β-cells treated with serum from patients with type I diabetes (McConkey and Orrenius, 1997). Recently, improved techniques and probes have re-established a role for calcium in apoptosis signalling (He *et al.,* 1997) but the mechanism remains unclear. Outflow from intracellular stores

and influx of Ca^{2+} across the plasma membrane could result in a sustained Ca^{2+} increase that acts as a signal for apoptosis. Depletion of $[Ca^{2+}]^i$ from the ER, thought to be an early event in the apoptotic pathway, has been shown to be sufficient to induce apoptosis by causing the subsequent depletion of Ca^{2+} from mitochondrial stores (Baffy *et al.,* 1993, He *et al.,* 1997). Anti-apoptotic members of the Bcl-2 family (discussed in section 1.3.3.4) have been shown to regulate Ca^{2+} compartmentalization in both ER and mitochondria (Baffy *et al.,* 1993, He *et al.,* 1997). Constitutive levels of C^{2+} in mitochondria were shown to be significantly lower in a Bcl-2-expressing haemopoietic cell line (32D) compared to vector control transfectants, supporting the idea that Bcl-2 regulates Ca^{2+} in mitochondria (Baffy *et al.,* 1993). The exact mechanism by which Bcl-2 regulates Ca^{2+} is currently unclear. One possible model is that Bcl-2 prevents the opening of Bax-induced channels, preventing Ca^{2+} efflux from the mitochondria.

A growing family of apoptotic proteases, the caspases are generally considered to be the executioners of apoptosis. The caspases are discussed in depth in Chapter 2. Once the caspases have enzymatically broken down the intercellular components, the cell fragments form apoptotic bodies and are destroyed by phagocytosis.

1.3.3.3 Signalling for apoptosis by ceramide

Ceramide is a secondary messenger liberated by hydrolysis of membrane sphingolipids (Ballou *et al.,* 1996, Hale *et al.,* 1996). The generation of ceramide following an apoptotic stimulus results in the activation of a protein kinase cascade (Anderson, 1997). A wide range of apoptotic stimuli have been demonstrated to lead to the generation of ceramide. Activation of TNFα and Fas activate sphingomyelinases resulting in the production of ceramide and apoptotic cell death (Ballou *et al.,* 1996). Apoptosis induced by γ-irradiation was also demonstrated to involve ceramide signalling using an endothelial cell line. Accumulation of ceramide was observed within minutes of irradiation and cell permeable ceramides were shown to be capable of causing apoptosis in these cells (reviewed in Ballou *et al.,* 1996). In contrast to TNFα, Fas and γ-irradiation, apoptosis induced by the DNA damaging agent daunorubicin was demonstrated to induce ceramide but without sphingomyelinase activation. Daunorubicin-induced apoptosis was shown to involve *de novo* synthesis of ceramide via stimulation of ceramide synthase, which was further supported by the use of inhibitors of ceramide synthase that blocked daunorubicin induced apoptosis (reviewed in Ballou *et al.,* 1996). A role for ceramide in apoptosis was reinforced by the observation that resistance to certain types of apoptosis was associated with insufficient production of ceramide (Gottschalk *et al.,* 1995).

The downstream events of ceramide production and their link to the signalling of apoptosis are relatively unknown. Ceramide is demonstrated to activate both the MAP kinase (mitogen-activated protein kinase) and the SAP kinase (stress-activated protein kinase) pathways (Canman and Kastan, 1996, Anderson, 1997). The MAP kinase pathway is thought to be involved in cell survival and growth whereas the SAP kinase pathway is thought to have

the opposing effect and has been associated with cell death (Canman and Kastan, 1996, Anderson, 1997). Therefore, the apoptotic response of ceramide may be as a result of the perturbation of these two opposing pathways in favour of the SAP kinase pathway, culminating in the apoptotic death of the cell (Canman and Kastan, 1996, Anderson, 1997).

1.3.3.4 The Bcl-2 family

Bcl-2, the acronym for the B-cell lymphoma/leukaemia-2 protein, was first discovered because it is overexpressed in follicular lymphoma and other B-cell malignancies (Tsujimoto *et al.,* 1985). Following a seminal report showing that Bcl-2 could synergize with c-myc to transform cells (Vaux *et al.,* 1988), Bcl-2 became the focus of much study (Reed, 1997). Bcl-2 is the founding member of a rapidly expanding family which include death antagonists (Bcl-2, Bcl-XL, Bcl-W, Bfl-I, Brag-1, Mcl-1 and AI) and death agonists (Bax, Bak, Bcl-xs, Bad, Bid, Bik and Hrk) (Reed, 1994, Kroemer, 1997). We therefore review the Bcl-2 family in its entirety here, but refer again to the protective effects of the Bcl-2-like members of the family in section 1.3.

The involvement of the Bcl-2 family in apoptosis is supported by studies of mice deficient for a particular Bcl-2 family member. Mice which are null for the anti-apoptotic Bcl-2 protein can be observed to be lymphopenic, azotemic and hypopigmented; all of these characteristics resulting from increased cell death (Veis *et al.,* 1993, Knudson and Korsmeyer, 1997). Bcl-XL deficient mice are embryonic lethal, displaying increased apoptosis in both haematopoietic and neuronal lineages (reviewed in Knudson and Korsmeyer, 1997). In contrast, mice deficient for the pro-apoptotic protein Bax demonstrate an excess of lymphocytes, granulosa cells and spermatogonia and have some neurons that display marked resistance to neurotrophic factor deprivation (reviewed in Knudson and Korsmeyer, 1997). Mice which have the genes for both *bcl-2* and *bax* knocked out had equivalent numbers of thymocytes compared to the control mice, illustrating that the lymphocyte loss observed in Bcl-2 null mice depends on Bax (reviewed in Knudson and Korsmeyer, 1997).

The family possess variable amounts of Bcl-2 homology (BH) regions (BH1 to BH4), governing their capacity to interact with each other or with other unrelated proteins (reviewed in Kroemer, 1997). The ratio of death antagonists to agonists may determine whether a cell will respond to an apoptotic signal. This life-death balancing act is believed to be mediated, at least in part, by competitive dimerization between selective pairs of antagonists and agonists (reviewed in Kroemer, 1997). Whether one group can neutralize the functions of the other is not clear since the family members may act independently of one another (Knudson and Korsmeyer, 1997).

Most members of the Bcl-2 family possess a carboxy-terminal transmembrane region which acts to tether them into internal membranes of the ER, mitochondria and nucleus (Krajewski *et al.,* 1993, Kroemer, 1997). However some family members such as Bid and Bad do not possess this hydrophobic region and are located in the cytoplasm. The localization

of Bcl-2 to membranes other than the mitochondria may neutralize the proapoptotic Bcl-2 family members, thereby preventing them from acting on the mitochondria (Kroemer, 1997). This theory is supported by the observation that ER-targeted Bcl-2 interacts with Bax, which itself must act on mitochondria to kill cells (reviewed in Kroemer, 1997). Alternatively, the effects of the Bcl-2 family may be mediated through the ER and nuclear membranes and not just through the mitochondria (reviewed in Kroemer, 1997). This theory is supported by the observation that ER-targeted Bcl-2 is capable of suppressing apoptosis induced by c-myc overexpression and serum withdrawal but not that induced by etoposide. In contrast, mitochondria-targeted Bcl-2 is an efficient inhibitor of apoptosis induced by all these stimuli, suggesting that Bcl-2 is a more efficient inhibitor of apoptosis when targeted to the mitochondria (reviewed in Zamzami *et al.,* 1998).

The anti-apoptotic proteins Bcl-2 and Bcl-XL prevent release of cytochrome c from mitochondria and the subsequent activation of caspase-3 (Yang *et al.,* 1997, Hengartner, 1998) (Chapter 2, Figure 2.1). Overexpression of Bax has been shown to result in its localization to the mitochondria and to induce the release of cytochrome c (Rosse *et al.,* 1998). In cells over-expressing Bcl-2, microinjection of cytochrome c no longer induces apoptosis (Zhivotovsky *et al.,* 1998). Taken together, these data suggest that Bcl-2 can regulate cytochrome c release from mitochondria but can also interfere with Bax mediated cell killing downstream of and independently of cytochrome c release (Rosse *et al.,* 1998). Several hypotheses have been put forward to explain these observations: Bcl-2 may bind directly to the Apaf complex or to cytochrome c in the cytosol to quench its effects (Zhivotovsky *et al.,* 1998).

Although Bcl-2 family proteins fail to show significant amino-acid sequence homology with other proteins, the three-dimensional structure of Bcl-XL appears to be similar to the pore-forming domains of certain bacterial toxins that act as channels for ions or proteins, such as diptheria toxin (Muchmore *et al.,* 1996). Thus, the Bcl-2 family of proteins may function as channels for ions, proteins or both (reviewed in Reed, 1997) and both Bcl-XL and Bcl-2 possess ion-channel activity in synthetic lipid membranes (Minn *et al.,* 1997, Reed, 1997). However, the pore formation is only observed at low pH (pH 4.0 or 5.5 for Bcl-XL and Bcl-2, respectively), suggesting that protonation of these proteins is required for pore formation. Recently, it has been demonstrated that Bax also exhibits channel activity *in vitro* (Zamzaini *et al.,* 1998) but the existence and function of pore formation *in vivo* at physiological pH remains unclear (reviewed in Zamzami *et al.,* 1998). The anti- and pro-apoptotic pores may have differential selectivity, providing either conduit, charge or direction specific transport of ions or molecules that regulate apoptosis (reviewed in Reed, 1997). Circumstantial evidence suggests that overexpression of Bcl-2 inhibits the mitochondrial permeability transition whereas Bax overexpression induced it (reviewed in Reed, 1997).

As well as forming pores, Bcl-2 and Bcl-XL can bind to a vast range of proteins including the protein kinase Raf-1, the protein phosphatase calcineurin, the GTPases R-Ras and H-

Ras, Nip-1, -2, -3 and a protein called BAG-I (reviewed in Reed, 1997). The binding of these signalling proteins provides a second function for the Bcl-2 family by acting as signal adapters or docking proteins.

The involvement of Bcl-2 in the Fas pathway is controversial and appears to depend on the experimental model (Itoh *et al.,* 1993, Chiu *et al.,* 1995, Lacronique *et al.,* 1996). Mice expressing human Bcl-2 in hepatocytes were protected against apoptosis induced by agonistic anti-Fas antibody (Lacronique *et al.,* 1996) and Bcl-2 down-regulation may be the first step in Fas-mediated apoptotic process in the male mouse reproductive tract following gonadectomy (Suzuki *et al.,* 1996).

Given the functional importance and central role of Bcl-2 related proteins in apoptosis control, they constitute prime targets for therapeutic interventions on numerous disease states mediated by dysregulated cell death. Knowledge of the mode of action of Bcl-2 related proteins may provide us with information for the development of new treatments and toxicological tests, as well as for the induction of apoptosis in tumour cells.

1.4 SUPPRESSION OF APOPTOSIS INDUCED BY TOXICANTS

1.4.1 Survival signals

As originally suggested by Raff (1992), cells must receive survival signals to avoid apoptosis. Survival signals comprise receptor binding of soluble growth factors such as interleukin-3 (IL3), cell-cell communications such as gap junctions and interaction with extracellular matrix components. It is not clear whether the various survival stimuli share common downstream signalling elements to suppress the default apoptotic pathway or whether they signal survival independently.

The mechanism by which growth factors permit cell survival is an area of great interest. It was found that signalling through Ras, Raf, and Src pathways, although mitogenic, was not sufficient to permit cell survival in fibroblasts (Kennedy *et al.,* 1997). One molecule which was found to be involved in the signalling of survival factors mediated by a wide range of growth factors is phosphoinositide 3 kinase (PI3K). Blockade of PI3K activity can suppress the ability of trophic factors to promote survival; conversely, in many cell types PI3K activity is sufficient to promote survival (Datta *et al.,* 1997) (see also Chapter 8, section 8.4.4.2). One target of PI3K found to be central to the delivery of survival signals is the serine-threonine kinase c-Akt, also known as protein kinase B (PKB). Activation of Akt is known to deliver a survival signal that inhibits apoptosis induced by a variety of stimuli, including growth factor withdrawal, cell-cycle discordance, loss of cell adhesion, and DNA damage (reviewed in Datta *et al.,* 1997). Akt activation results in the inhibition of caspase activity to protect from apoptotic cell death (Del Peso *et al.,* 1997). One possible mechanism by which PI3K/Akt suppresses apoptosis and promotes cell survival is via

modulation of members of the Bcl-2 family. This was supported by the observations that activation of Akt in cells resulted in the phosphorylation and inactivation of the pro-apoptotic Bcl-2 family member, Bad (Datta *et al.,* 1997, Del Peso *et al.,* 1997). Activation of PI3K/Akt does not result in changes in either Bcl-2, Bcl-XL or Bax (Kennedy *et al.,* 1997). Therefore, Bad phosphorylation by Akt is one of many possible mechanisms by which survival signals may result in the suppression of apoptosis.

In addition to soluble factors, cell-cell contact and microenvironment plays an important role in cell survival (Bates *et al.,* 1994). Within the germinal centre, ligation of the cell surface antigen CD40 sends a signal to maturing B cells that is necessary to prevent their death by apoptosis (Wang *et al.,* 1997). The PI3K/Akt pathway and the Bcl-2 family have been implicated in the survival pathways. Interestingly, CD40 signalling also suppresses drug-induced apoptosis in B lymphoma cells (Walker *et al.,* 1997) increasing the probability of relapse after chemotherapy. Extracellular matrix also determines cell survival since epithelial cells from the mammary gland (Pullan *et al.,* 1996) survive only when attached to the correct extracellular matrix; inappropriate or inadequate matrices resulted in the death of the cells, even in the presence of cell-cell contacts and soluble trophic factors. In summary, survival factors and the extracellular environment is of major importance with respect to the control of apoptosis and the outcome of exposure to toxicants.

1.4.2 The Bcl-2 family

There have been a multitude of reports showing that enforced overexpression of Bcl-2 or of Bcl-XL acts to delay the onset of apoptosis induced by toxicants (Fisher *et al.,* 1993, Schott *et al.,* 1995). The vast majority of these reports concentrate on apoptosis induced by DNA damaging agents in cancer cell lines and conclude that Bcl-2 or Bcl-XL renders the cells drug resistant. However, most of these studies assess short-term survival and overlook subsequent clonogenicity, questioning their relevance to long-term cell survival. In the few studies that consider clonogenicity, Bcl-2 overexpression can give a clonogenic advantage after drug induced damage (Walker *et al.,* 1997), allowing cells with DNA damage to survive and proliferate.

1.5 SUMMARY

The maintenance of tissue homeostasis is a fine balance between cell survival and cell death. Since apoptosis appears a common response to many toxicants, it has become a particularly hot topic for toxicologists. Recent research has identified key molecules involved in the regulation of apoptosis and these (and others yet to be identified on the apoptotic pathways) are now major sites for investigation by toxicologists with a view to therapeutic intervention.

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CHAPTER 2

Toxicological Consequences of Caspase Inhibition and Activation

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Contents

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2.2 Enzymology of caspases
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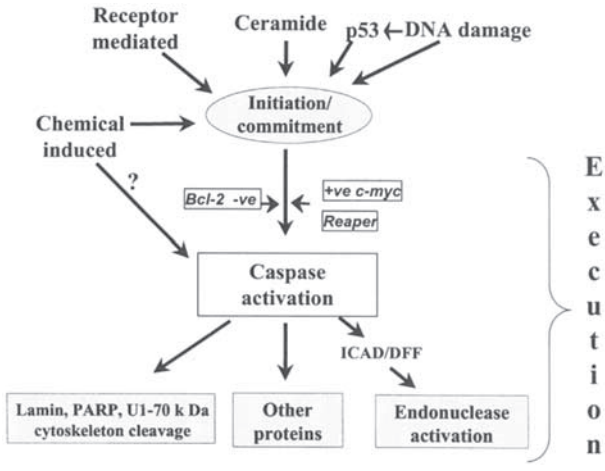

Figure 2.1 A schematic diagram depicting the pathways of apoptosis, showing the pivotal role played by caspases in the execution of cell death.

2.1 INTRODUCTION

Since the original finding by Kerr *et al.* (1972) which described the morphology of apoptotic cell death, considerable progress has been made in unravelling the biochemistry and molecular biology of the process (reviewed in Hale *et al.,* 1996, Adams and Cory, 1998, Ashkenazi and Dixit, 1998, Evan and Littlewood, 1998, Green, 1998, Green and Reed, 1998, Thornberry and Lazebnik, 1998). These pathways have been described in detail in Chapter 1 and are summarized in Figure 2.1. Irrespective of how cell death is initiated, death signalling proceeds to an execution stage that involves caspase activation and the subsequent proteolytic dismembering of the cell. The toxicological consequences and mechanisms of this caspase activation and its inhibition are considered in this chapter.

The activation of the caspases seems to represent an all or nothing, one way process concluding in life or death of the cell. Thus, the possible toxicological consequences of interference with caspase activity are great and could be derived from either inappropriate activation or inhibition of the caspases. To address this, the enzymology of the caspase enzymes is discussed in the next section followed by a final section describing modulation of caspase activity and the possible consequences of activating or inhibiting caspases.

2.2 ENZYMOLOGY OF CASPASES

2.2.1 General properties

Caspases were first implicated in apoptosis when CED-3, a protein required for programmed cell death in *Caenorhabditis elegans,* was found to have close homology with the

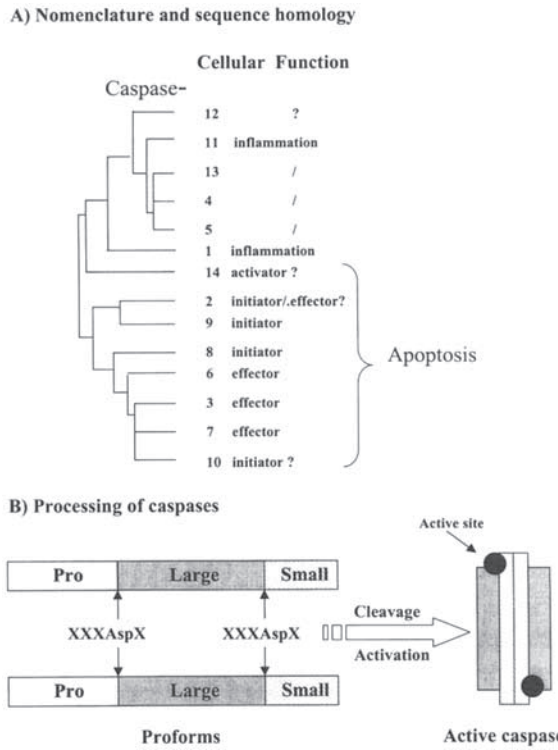

Figure 2.2 (A) Nomenclature and sequence homology for the caspase family.
(B) Schematic diagram depicting the processing of caspases from a pro-form to the active form.

mammalian interleukin-1β-converting enzyme (ICE or caspase-1) and that overexpression of ICE-induced apoptosis (Miura *et al.,* 1993, Yuan *et al.,* 1993). Since this initial discovery there has been an avalanche of papers describing new CED-3 homologues (reviewed in Cohen, 1997). This rush of papers inevitably led to a plethora of acronyms for the new homologues, often with duplication. In order to rationalize this confusing picture, a uniform nomenclature was proposed and now seems to be universally accepted (Alnemri *et al.,* 1996). Thus, the generic name for all family members is caspase with the *c* denoting a cysteine protease and *aspase* referring to the aspartate specific cleaving ability of these enzymes. The individual members are then numbered according to their chronological order of publication and thus ICE became caspase-1. Additional descriptives for the main functions are now quite commonly used such as initiator/activator or effector caspases.

To date 14 caspases have been identified (12 human and 2 murine) and are listed in Figure 2.2, which depicts the phylogenetic and probable cellular functions (Thornberry

and Lazebnik, 1998). Caspases have marked similarities in amino acid sequence, structure and substrate specificity (Cohen, 1997, Thornberry and Lazebnik, 1998). They are synthesized as pro-enzymes with molecular weights ranging from 30–50 kDa and contain three domains: a N-terminal pro-domain which varies considerably between family members, a large subunit (~20 kDa) and small subunit (~10 kDa) domain which show considerable homology between family members. The pro-caspases are believed to be essentially, catalytically inactive (Thornberry and Lazebnik, 1998, but see later) and must be cleaved at specific XXXAsp↓X sites to yield the large and small subunits which assemble as the active 2 X heterodimer. The active caspases cleave proteins at unique tetrapeptide motifs (NH_2—P_4-P_3-P_2-P_1—COOH) and have an absolute specificity for aspartic acid in the P_1 position. Crystallographic studies on caspase-1 and caspase-3 (Wilson *et al.,* 1994, Mittl, 1996) show that the tetrameric form is a sandwich-like structure in which the two small subunits are in association with one another and bounded by the large subunits (Figure 2.2B). Each large/small subunit pair shares amino residues to form an active site, which contains the critical catalytic cysteine (Cys-238), required for caspase activity. All the caspases contain an active site pentapeptide motif QACXG (where X can be R, Q or G) which contains the catalytic cysteine. In the case of caspase-1 the Asp pocket (P_1) is formed from residues Arg-179, Gln-238, Arg-341 and Ser-347.

Caspase are relatively specific proteases and a positional-scanning combinatorial substrate approach (Thornberry *et al.,* 1997) has been used to characterize the caspases on the basis of their substrate specificities into three functional subgroups (Figure 2.2). Group I caspases (-1, -4 and -5) cleave at W/LEHD tetrapeptide motifs. The function of the group-1 caspases in apoptosis is unclear (reviewed in Cohen, 1997) and it is more likely that there main cellular function is in the production of the inflammatory response. The effector caspases (Group II) which include -2, -3 and -7 (and also CED-3) cleave the DEXD tetrapeptide motif which is found in many proteins associated with apoptosis (Table 2.1). Group III (activator) caspases include caspases-6, -8 and -9 as well as granzyme B and cleave at (I, V, L) EXD tetrapeptide sequences. The latter group of caspases are so-called because they are believed to be responsible for processing and activating the effector caspases. While this provides a rational approach to classifying the caspases, it cannot be regarded as an absolute definition of caspase functionality. Caspase-6, for example, on the basis of its substrate specificity would be predicted to be an activator caspase (Thornberry *et al.,* 1997). However, there is good evidence (Takahashi *e*t *al.,* 1996) to suggest that this is the caspase responsible for lamin cleavage and the dismantling of the nuclear envelope (Table 2.1) and as such should be regarded as an effector caspase.

2.2.2 Activation and initiation of the caspase cascade

The activation of the caspase cascade, whether it be during receptor or non-receptor (chemical) induced-apoptosis, requires that the activator caspases are processed and

TABLE 2.1
Proteins cleaved by effector caspases and their functions

Protein target	Cleavage motif(s)	Caspase(s)	Biochemical activity of target
PARP*	DEVD↓G	3, 7	DNA repair
U1-70 kDa	DGPD↓G	3	RNA splicing
DNA-PK_{cs}	DEVD↓N	3	DNA double strand-break repair
Lamin-A	VEID↓N	6	Essential for nuclear membrane integrity
ICAD/DFF	DETD↓S;DAVD↓T	3	Activation of DNAse
Gas2	SRVD↓G	?	Element of microfilament system
Protein kinase C δ	DMQD↓N	3	Activated during apoptosis
SREBP-1	DEPD↓S	3, 7	Sterol regulatory element binding proteins
SREBP-2			
Huntingtin	DXXD↓S	3	Huntington disease gene product
Fodrin	DETD↓S	?	Cytoskeletal protein

*poly (ADP ribose) polymerase

activated. For an activator caspase to process an effector caspase, it must first be activated itself. The key question is how is this achieved? In the case of receptor mediated apoptosis (see Ashkenazi and Dixit, 1998 for review) there is considerable evidence to suggest that during Fas/Apol/CD 95 induced apoptosis the apical caspase-8 is activated by association with the receptor itself. The Fas receptor which is a member of the tumour necrosis actor (TNF) receptor gene superfamily, is characterized by a cysteine-rich extracellular region, a transmembrane domain and a homologous cytosolic region known as the death domain (DD). Trimerization of the receptors occurs when the Fas ligand engages with its receptor which then binds an adaptor protein, FADD (Fas-associated death domain) which also has a similar death domain to the death domains of the trimerized receptor (Figure 2.3). FADD also contains a death effector domain (DED) sequence which binds to an analogous (one of a tandem repeat) region on pro-caspase-8. Both pro-caspase-8 and pro-caspase-10 contain tandem DED sequences, which are basically protein-protein interaction domains and are similar, but unrelated to the caspase recruitment domains (CARDS) of caspases-1, -2, -4, -5, and -9 (Takahashi *et al.,* 1996). The recruitment of pro-caspase-8 to the receptor is believed to result in oligomerization and autocatalytic processing of caspase-8 to its active form (Muzio *et al.,* 1998, Yang *et al.,* 1998a). This induced proximity or oligomerization model of caspase activation provides, if correct, an elegant explanation for activator caspase activation. As discussed by Thornberry and Lazebnik (1998) this hypothesis is based on the fact that pro-caspase-8 has low (1–2% of the processed enzyme) proteolytic activity and that when artificially forced to oligomerize, it auto/transprocesses into its active form and causes cell death (Muzio *et al.,* 1998, Yang *et al.,* 1998a). However, there is as yet no

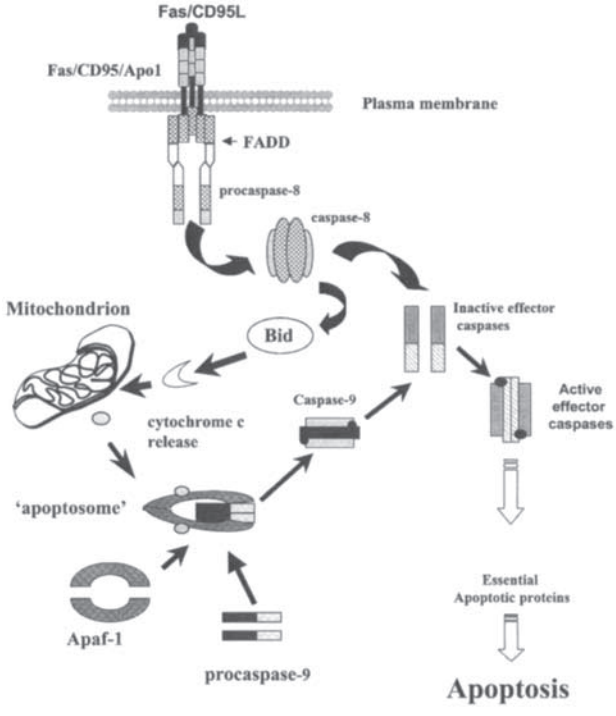

Figure 2.3 Caspase activation during receptor and non-receptor mediated apoptosis.

evidence that pro-caspases exist as monomers and also it is unclear as to how the caspase catalytic active site could be formed in the intact pro-enzyme or after oligomerization occurs. Validation of this model of caspase activation thus requires the structure of the pro-caspase molecule to be solved.

In the case of non-receptor (chemically) mediated caspase activation, there is considerable evidence to suggest that additional co-factors are needed to activate the caspase cascade. Three apoptotic protease-activating factors have been identified which are required for caspase-3 activation (Liu *et al.,* 1996, Li *et al.,* 1997, Zou *et al.,* 1997). Apaf-1 is the first identified mammalian homologue of CED-4. It is a large (Mr=130 kDa) soluble protein with a short N-terminal CARD domain, a central CED-4 homology domain followed by twelve WD-40 repeats, which are probably involved in protein-protein interactions. Apaf-1 in the presence of dATP, complexes with Apaf-2 (cytochrome c) and Apaf-3 (pro-caspase-9) to form an 'apoptosome' (Hengartner, 1997) in which caspase-9 is processed to its active form (Figure 2.3). The activated caspase then processes caspase-3 and initiates the caspase cascade. As caspase-9 has a DQLD↓A cleavage site (Srinivasula *et al.,* 1996) which can be cleaved by caspase-3, it is possible that activation of caspase-3 leads to further cleavage of caspase-9, thereby amplifying the caspase activation event.

Interestingly, caspase-9 also has a granzyme-B cleavage site (PEPD↓A) which suggests that caspase-9 can be activated in an Apaf-1 independent manner, during T-cell mediated apoptosis (Duan *et al.,* 1996). The mechanism by which the apoptosome complex activates caspase-9 remains to be elucidated, although evidence has been presented for a model in which Apaf-1 binds caspase-9 (through their respective CARD interaction domains) and then oligomerizes via the CED-4 homology domains, resulting in procaspase aggregation and activation (Srinivasula, 1998). Removal of the WD-40 repeats eliminates the requirement for cytochrome c, indicating that cytochrome c activates Apaf-1 by binding to the WD-40 repeat domain. Support for oligomerization of Apaf-1 as an intrinsic mechanism for caspase activation also comes from a recent report that in the C.elegans cell death model, CED-4 oligomerization is also required for CED-3 activation (Yang *et al.,* 1998b).

Thus, in receptor and non-receptor mediated caspase activation, a common event is the formation of a complex which then permits the autocatalytic activation of caspases. However, the two pathways differ significantly in that Apaf-1 mediated activation requires two additional co-factors, cytochrome c and dATP. Thus, factors which release cytochrome c and/or increase the concentration of dATP in the cell are potentially capable of activating caspases directly, a property which may have important toxicological and therapeutic consequences if caspase modulators are utilized as drugs (see later). The mechanism by which cytochrome c is released from mitochondria remains controversial but there is little doubt that it is a commonly observed feature in apoptosis. Significantly, cytochrome c release during non-receptor mediated apoptosis is not blocked by caspase inhibitors and appears to occur upstream of the caspase activation event (Bossy-Wetzel *et al.,* 1998). However, inhibition of caspase-8 activity during Fas-induced apoptosis prevents cytochrome c release (Vander Heiden *et al.,* 1997, Kuwana *et al.,* 1998). An explanation for this has been put forward with the finding that Bid, a BH3 domain-containing pro-apoptotic Bcl-2 family member, is cleaved during Fas-induced apoptosis by caspase-8 (Li *et al.,* 1998, Luo *et al.,* 1998). The C-terminal product then translocates to the mitochondria and causes cytochrome c release (Figure 2.3) which then activates the Apaf-1/caspase-9 pathway. These findings suggest an amplification role for caspase-3 in receptor-mediated apoptosis and demonstrates that there is cross-talk or overlap between receptor and non-receptor mediated caspase activation.

In addition to Fas-induced apoptosis, several other death receptors have been identified which on ligation and trimerization of the receptor result in caspase activation. These receptors are related to the TNF receptor and have an additional common adaptor protein TRADD (TNFR-associated death domain) which recruits RIP (receptor-interacting protein), TRAP 2 (TNF receptors associated factor 2) and FADD (Figure 2.4), thereby conveying a dual functionality for this type of receptor. Thus, FADD recruits caspase-8 and activates the caspase cascade while RIP/TRAF2 activate the NF-κB-inducing kinase (NIK), which in turn activates the inhibitor (I-κB) of the κB kinase complex (IKK). I-κB is then

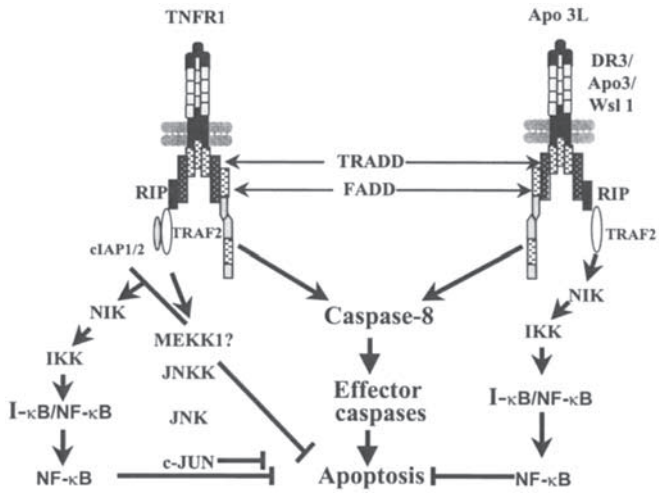

Figure 2.4 TNFα and DR3 receptor-mediated caspase activation.

phosporylated by IKK allowing the degradation of I-κB and the release of NF-κB which translocates to the nucleus where it initiates transcription and inhibits apoptosis. Thus, for TNFα to induce apoptosis it is usually necessary to inhibit protein transcription, thereby inactivating the NF-κB pathway and allowing the FADD/caspase-8 activating pathway to predominate. TRAF2 can also activate c-JUN by a cascade that includes mitogen-activated protein (MAP) kinases such as MEKK1 (MAP/Erk kinase kinase-1) and JNKK (JNK kinase) to inhibit apoptosis. FADD can also engage an adaptor called RAID or CRADD which binds via a death domain to RIP and via a CARD-CARD interaction to caspase-2, thereby inducing apoptosis (Ahmad *et al.,* 1997, Duan and Dixit, 1997).

Another TNF related ligand TRAIL (Apo 2L) has been identified (Pitti *et al.,* 1996) which binds to two receptors DR4 (Pan *et al.,* 1996) and DR5 (MacFarlane *et al.,* 1997). TRAIL is similar to Fas but its action, surprisingly, is not blocked by overexpression of a FADD dominant negative mutant (Marsters *et al.,* 1996).

2.3 MODULATORS OF CASPASE ACTIVITY

There are many natural and synthetic modulators that inhibit caspases either by directly inhibiting the enzyme or alternatively preventing the activation of the caspases. These will be discussed in this section. The major controlling factors for apoptosis appear to be upstream of caspase activation (see Chapter 1 for a discussion of commitment and execution). However, the existence of viral inhibitors suggest that the caspase-inhibited cell remains sufficiently viable for the virus to replicate itself even though the cell death

commitment status of the cell has not changed. It is not clear whether cell death has been temporarily delayed or abolished although there is some evidence that caspase inhibited cells still have clonogenic potential (Longthorne and Williams, 1997).

2.3.1 Viral inhibitors

Three distinct classes of viral inhibitors have been identified: Crm A, p35 and IAPs (Table 2.2). Cowpox virus Crm A is a potent inhibitor of some activator caspases, whilst the baculovirus p35 protein has no known homologues. Both Crm A and p35 need to be cleaved in order to inhibit the caspases. The IAPs present a more complex story, as they do have mammalian homologues. One of these, x-like IAP is a potent inhibitor of caspase-3 and -7 (Deveraux *et al.,* 1997), suggesting a direct inhibitory action for these proteins in non-receptor mediated apoptosis. However, in the case of receptor mediated apoptosis a more complicated picture emerges in which the human homologues c-IAP1/c-IAP2 bind to TRAF2 and regulate NF-γB activation (Chu *et al.,* 1997, Clem and Duckett, 1997) resulting in the inhibition of the apoptotic pathway. Whilst the mechanisms of action of the IAPs are still largely unknown it is clear that they have an evolutionary conserved regulatory role in apoptosis.

2.3.2 Ced-9 like proteins

The growing Bcl-2 family of proteins has an important role in apoptosis (reviewed in Adams and Cory, 1998, Green, 1998, Green and Reed, 1998), but it is only recently that some of the potential mechanisms have become apparent (Table 2.2). The pro-survival members, Bcl-2/Bcl-XL seem to act by either by preventing the release of cytochrome c from the mitochondria (Figure 2.3), abrogating activation of the apoptosome and/or by binding to constituent members of the apoptosome such as Apaf-1 to block caspase activation by preventing oligomerization. The pro-apoptotic members fall into two families (Adams and Cory, 1998) the Bax-like (includes Bak and Bok) family and the BH3 family (Bik, Blk, Hrk, BNIP3, Bim_L Bad, Bid, EGL-1). The latter family is distinguished by the fact that they contain the BH3 domain, which seems to be essential for promoting cell death by stimulating the release of cytochrome c and activating the caspases (Luo *et al.,* 1998, Figure 2.3). The Bax-like family members contain other Bcl-2 domains (BH1 and BH2) which are important in allowing homo- and hetero-dimerization with other Bcl-2 family members. These interactions probably play a direct role in the action of the pro-apoptotic Bax-like proteins by binding/neutralizing the anti-apoptotic effects of Bcl-2 or Bcl-XL. Irrespective of the precise mechanisms of action it is clear that the Bcl-2 family of proteins play a major role in regulating caspase activity.

TABLE 2.2
Modulators of caspase activity

Inhibitor	Specificity	Comments
(A) Natural inhibitors of caspase activity		
Crm A (serpin like inhibitor)	Caspase-1, -4, -8 and -6	Reversible inhibitor, mode of action in receptor mediated apoptosis probably involves cleavage of Crm A and binding of cleaved product to active site.
P35 (baculovirus gene product)	Caspase-1, -2, -3 and -4	Cleavage of p35 at DQMD↓G site produces inhibitory peptide and blocks receptor mediated apoptosis.
IAPs (XIAP, human X-linked)	Caspase-3, -7	Large family of viral inhibitors, only one known to have mammalian members; reversible inhibitor of caspases binds to active site.
IAPs (c-IAP1, c-IAP-2)	Bind to TRAF2	May modulate NF-κB activation during TNFα signalling and prevent activation of caspases.
Bcl-2/Bcl-xL	Non-receptor mediated apoptosis	Prevents release of cytochrome c during apoptosis but may also have other downstream effects by binding to Apaf-1 and caspase-9.
(B) Active site peptide inhibitors		
Ac-YVAD.CHO*	Caspase-1, -4 and -5	Reversible inhibitor, relatively specific and potent inhibitor (K_i = < 1–360 nM) but requires much higher (K_i > 10 μM) concentrations to inhibit effector caspases.
Ac-DEVD.CHO	Caspase-3, -7	Reversible inhibitor, with specificity for caspase-3 and -7 (K_i = 0.5 and 35 nM) but inhibits caspase-1 and -4 at only moderately higher concentrations (K_i = 15–135 nM).
-CMK, -FMK and AMK	All caspases	Covalent inhibitors, specificity determined by peptide sequences.
Z-VAD.FMK	All caspases	Irreversible inhibitor, tripeptide sequence means that it is less specific and will inhibit all caspases. Also methyl group on aspartic acid renders the inhibitor cell permeable.
(C) Decoy proteins		
vFLIPs and cFLIP (Casper/I-Flice/ MRIT Flame/ CASH/USURPIN)	FADD/caspase-8	Contain death effector domains similar to FADD and caspase-8. Role and mechanism of FLIPs controversial as it has been reported to both inhibit and activate apoptosis. But recently shown to heterodimerize with caspase-8 preventing recruitment by FADD.
Decoy receptor 1 (DcR1/TRID/ TRAIL-R3)	DR4 and DR5 receptors	Prevent activation of caspases by binding TRAIL ligand and preventing DR4 and DR5 receptors being ligated.
Decoy receptor 2 (DcR2/TRAIL-R4/TRUND)	DR4 and DR5 receptors	Contain truncated death mains and compete for TRAIL, thereby preventing DR4 and DR5 being ligated.

2.3.3 Synthetic active site-directed peptide based inhibitors

An important development in our understanding of caspases has been the use of peptide site-directed inhibitors that target a particular caspase depending on their peptide sequence. The inhibitors can be divided into either reversible or irreversible inhibitors depending on the chemical identity of the leaving group (X) on the C-terminal side of the aspartate cleavage site (Thornberry and Molineux, 1995). Thus, if X is an aldehyde, nitrile or ketone the inhibitor will undergo nucleophilic addition of the catalytic cysteine to form thiohemiacetals, thioimidates and thiohemiketals, respectively. These intermediates are believed to mimic the acyl-enzyme intermediates formed during enzyme hydrolysis, but unlike the substrate-enzyme ternary complexes, the dissociation of the inhibitor-enzyme complexes is very slow. The irreversible inhibitors usually employed are a-substituted ketones with the general structure R-CO-CH_2-X where X is a carboxylate (acyloxymethylketone), diazonium (diazomethylketone) or halogen (halomethylketone) leaving group (Thornberry and Molineux, 1995). The inhibitors inactivate the catalytic cysteine of the caspase to form a thiomethylketone. The irreversible inhibitors are generally described as being very specific, and in the case of caspase-1 like inhibitors (Ac-YVAD.CHO and Ac-WEHD.CHO) (Garcia-Calvo *et al.,* 1998) these are active against the caspases-1, -4 and -5 in the low nM range, but much higher concentrations (i.e>10 µM) are required to inhibit effector caspases -2, -3, -6 and -7. In contrast Ac-DEVD.CHO is much less specific even though it will inhibit caspase -3, -7 and -8 at low concentrations (K_I between 0.23–1.6 nM) it will inhibit caspase-1 and caspase-4 at only moderately higher concentrations (15–135 nM). It must also be stressed that these differences in specificity are observed *in vitro* assays where the time of incubation and the concentrations are very carefully controlled. Increasing the inhibitor concentration or incubation time will diminish the apparent specificity of the inhibitor.

Many of the peptide inhibitors mentioned above are not particularly cell-permeable. However, Z-VAD.FMK (benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone by virtue of its benzyloxycarbonyl and OMe groups is cell permeable and therefore is very good at blocking apoptosis. Also, as it is irreversible and less specific (by virtue of its tripeptide motif) it can be used to inhibit all caspases in a wide variety of systems (Cohen, 1997, Villa *et al.,* 1997).

2.3.4 Natural decoy proteins

A final class of caspase inhibitors works in an indirect manner by preventing activating of the caspases during receptor-mediated apoptosis. These inhibitors function either by preventing recruitment of FADD such as cFLIP (Rasper *et al.,* 1998) or by binding the death effector ligand to act as decoy receptors (Pan *et al.,* 1997). The distribution of the decoy and functioning receptor can vary from tissue to tissue and also between normal

and cancer cells (Ashkenazi and Dixit, 1998). Thus, this balance between the decoy and functioning receptor may determine whether or not caspase activation and cell death are triggered.

2.4 TOXICOLOGICAL CONSEQUENCES OF CASPASE INHIBITION AND ACTIVATION

To date, there are no data on the use of direct inhibitors or inducers of caspase activity. Thus, an assessment of the consequences of the use of such agents must be speculative. The putative use of caspase inhibitors relies on the development of compounds that can selectively target caspases to inhibit apoptosis *in vivo*. In this respect, any experimental drug design will almost certainly be based on peptide inhibitors; it is unlikely that a compound could be synthesized that would be specific for only one caspase. Thus, any use of such a compound raises the possibility of unwanted caspase inhibition with specificity of action and toxicity determined by the pharmacokinetic profile of the drug.

There is good experimental evidence from animal studies to show that caspase inhibitor compounds can protect against excessive apoptosis in the whole animal. When anti-Fas antibody is injected into mice, it produces a rapid and fatal liver failure (Ogasawara *et al.,* 1993) and death of the animal. This can be abrogated by administration of Z.VAD.FMK (Rodriquez *et al.,* 1996, Kuenstle *et al.,* 1997, Chandler *et al.,* 1998) or YVAD.CMK before or after the injection of the anti-Fas antibody (Rouquet *et al.,* 1997). Both Z-VAD.FMK and YVAD.CMK can also block the liver damage induced by TNFα (Rodriguez *et al.,* 1996, Rouquet *et al.,* 1997). These experiments showed that there was a critical time period, during which the Z-VAD.FMK could be administered, in order to abrogate the liver injury. Furthermore, the protective effect of Z-VAD.FMK treatment seemed to be long-lived as the plasma levels of liver enzymes remained normal 4 days after the Z-VAD.FMK injection. These experiments clearly demonstrate that caspase inhibitors can be remarkably effective at attenuating the toxicological consequences of caspase activation and caspase inhibition can apparently rescue cells that are committed to die. However, this, raises a contentious point; several studies (Brown *et al.,* 1997, Amarante-Mendes *et al.,* 1998, Friedlander and Yuan, 1998) have reported that Z-VAD.FMK can block non-receptor mediated apoptosis but does not confer clonogenic viability. Thus, in these experiments, Z-VAD.FMK may act downstream of the commitment point so that cells can still die but by a slower non-apoptotic form of cell death (Ashkenazi and Dixit, 1998). However, Z-VAD.FMK has been reported to maintain clonogenic potential of Jurkat cells (Longthorne and Williams, 1997). A possible explanation for these differences is that in chemical, non-receptor mediated cell death the mitochondrial effects upstream of caspase activation are sufficient in themselves to kill the cell (Ashkenazi and Dixit, 1998). Such a mechanism would not operate in receptor mediated apoptosis since caspase-8 activation is needed for the secondary downstream mitochondrial effects.

Additionally, there is good evidence that, *in vivo,* caspase-inhibited cells can recover and switch from a 'committed to die' status to a 'committed to live' status. For example, caspase inhibition has been shown to ameliorate a number of neurodegenerative diseases (reviewed in Friedlander and Yuan, 1998).

There is thus clear experimental evidence that inhibiting caspases either by drugs or genetic manipulation can help to limit or abrogate pathological lesions that involve apoptosis. In these *in vivo* models the rescued cells survive and may proliferate to be replaced by new cells in a controlled and non-damaging manner. This raises the possibility that anti-caspase treatment can in itself lead to toxicity. In most studies where caspase inhibitors have been injected there have been no reported incidences of acute toxicity but data on chronic toxicity are not yet available. Clearly, there need to be long-term studies on caspase inhibitors to assess whether or not these anti-caspase compounds agents can act as non-genotoxic carcinogens (see Chapter 8).

Chronic dosing with caspase inhibitors could result in dysfunction in those tissues where apoptosis is vital to function. For example, the deletion of self-reacting cells during development of the immune repertoire could be inhibited. Evidence from knockout mice studies provide evidence of possible toxicity. As shown in Table 2.3, knockout mice have been generated for caspase-1, -2, -3, -8, -9 and Apaf-1. In the case of the group I caspases, 1 and 2, the animals develop normally to adulthood. The main defect in caspase-1 knockouts is related to inflammatory reactions, demonstrating a minimal role for caspase-1 in apoptosis. The caspase-2 knockout mouse provides evidence for a role in germ cell deletion and also a sensitization of neuronal cells to apoptosis. The effect of knocking out effector and activator caspases (-3, -8 and -9) and Apaf-1 is dramatic, producing marked embryonic malformations and lethality. Apaf-1 and caspase-9 show marked brain abnormalities and non-receptor mediated (mitochondrial) apoptosis is blocked without affecting cytochrome c release. Thymocytes from these mice still respond to Fas, demonstrating the different pathways involved in receptor mediated caspase activation. The caspase-8 embryos exhibit *in utero* lethality with heart and haemopoietic defects and cells derived from these animals exhibit normal non-receptor mediated apoptosis. These knockout studies are valuable in that they clearly point to different caspase requirement in different tissues at different stages of development. Thus, non-receptor mediated (mitochondrial dependent) apoptosis which requires Apaf-1 and caspase-9 is clearly essential for normal brain development, whilst caspase-8 and receptor mediated apoptosis would appear to be essential for the cardiovascular system. These findings also show that blocking caspase activation *in vivo* leads to excess numbers of cells being retained; cells live even though the death commitment point has been passed.

Another useful example of caspase inhibitor-mediated toxicity comes from Chinnaiyan *et al.,* 1997 and Yoshida *et al.,* 1998 who studied the possible use of caspase inhibitor in AIDS therapy. In this disease, accelerated apoptosis contributes to the $CD4^+$ T-cell deletion and it had been proposed that inhibiting cell death might provide a

TABLE 2.3
Phenotype of caspace and Apaf-1 knockout mice

Knockout genotype	Phenotype of mice	Reference
Caspase-1$^{-/-}$	• cannot generate IL-1β, but develop normally and are fertile with no gross abnormalities in apoptosis • resistant to LPS-induced endotoxic shock defects mainly in inflammatory system • protects against neuronal apoptosis in double transgenic model	Brown *et al.*, 1997 Kuida *et al.*, 1995
Caspase-2$^{-/-}$	• develop normally to adulthood and no obvious abnormalities • excess numbers of germ cells and oocytes resistant to chemotherapeutic drugs • B lymphoblasts resistant to granzyme B and perforin • sympathetic neurones more sensitive to NGE withdrawal	Bergeron *et al.*, 1998
Caspase-3$^{-/-}$	• die at 1–3 weeks of age, brain mass higher, hyperplasia and failed neuronal apoptosis • no noticeable defects in other tissues • caspase-3 plays a dominant non-redundant role in neuronal development	Kuida *et al.*, 1998
Caspase-8$^{-/-}$	• lethal *in utero* as embryos had impaired heart development and congested accumulation of erthyrocytes • fibroblasts derived from embryos non-responsive to TNFα, Fas and DR3 induced cell death • non-receptor mediated cell death not affected • caspase-8 plays a non-redundant role in embryonal development	Varfolomeev *et al.*, 1998
Caspase-9$^{-/-}$	• embryonic lethality, defective brain development • embryonic stem cells and fibroblasts resistant to several apoptotic stimuli • thymocytes insensitive to non-receptor mediated apoptosis but cytochrome c release not affected • thymocytes sensitive to UV and CD95 induced apoptosis	Hakem *et al.*, 1998 Kuida *et al.*, 1998
Apaf-1$^{-/-}$	• embryonic lethality with striking craniofacial lesions and brain abnormalities • embryonic stem cells resistant to non-receptor mediated apoptosis even though cytochrome c release unimpaired • thymocytes still respond to Fas-mediated killing • Apaf-1 is essential for mitochondrial related cell death	Cecconi *et al.*, 1998 Yoshida *et al.*, 1998

therapeutic treatment for HIV infection. However, this study showed that treating peripheral blood mononuclear cells (PBMCs) with Z-VAD.FMK enchanched HIV replication. Further more, Z-VAD.FMK treatment of PBMCs isolated from asymptomatic patients stimulated endongenous virus production. These findings vividly illustrate the possibly inadvertent harmful effects of using inhibitors as therapeutic agents.

Finally, there are many anti-cancer agents that induce apoptosis and caspase activation.

To date, there has been little evidence of chemicals inducing apoptosis by directly activating the caspases. However, the discovery of Apaf-1 and the role of dATP and cytochrome c suggest that direct activation of caspases could occur and there is a considerable amount of evidence that adenosine and ATP can induce apoptosis possibly via interaction with purinoceptors (reviewed in Chinnaiyan *et al.,* 1997). However, a recent study with fludarabine (2-chloro-2'-deoxyadenosine, 2CdA) has shown that adenosine analogues can kill cells in a much more direct manner (Francheschi *et al.,* 1997). Fludarabine is an anti-metabolite which induces apoptosis in quiescent lymphocytes and is used in treating lymphoproliferative diseases. The fact that an anti-metabolite can induce apoptosis in non-cycling cells is surprising but recent studies have shown that fludarabine is phosphorylated to 2CdATP which then activates caspases by interacting with Apaf-1 and cytochrome c. ATP depletion can also prevent caspase activation stimulated by anticancer agents, implicating the Apaf-1/cytochrome c pathway for caspase activation (Leoni *et al.,* 1998). These results demonstrate that caspase activation and cell death can be induced directly and perhaps explains why these compounds are so toxic.

2.5 SUMMARY

Caspases are the executioners of cell death and their activation and subsequent actions are tightly controlled. Precise metabolic pathways exist to activate the caspases, which then dismantle the cell. Ultimately, most death-inducing signals result in caspase activation and, as such, the caspases represent a common checkpoint for controlling cell death. *In vivo* studies strongly suggest that blocking caspase activity allows a cell to survive and the consequences of unwanted cells surviving can be dramatic and occasionally lethal to the organism. Equally damaging, however, is the inappropriate activation of caspases. Thus, there will be continued interest in developing drugs which can modulate caspase activity. However, the toxicological consequences of long-term anti-caspase treatments are completely unknown and need further study.

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CHAPTER 3

Modulation of Apoptosis in Immunotoxicology

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3.1 INTRODUCTION

Certain microbial or chemical toxicants affect the immune system. They are immunotoxicants in that they are able to cause acute or chronic disease by means of suppression or overstimulation of the immune system. Anergy (the inability to mount an immune response to a particular antigen) and depletion of effector cells such as T lymphocytes or macrophages are characteristic of immunosuppression induced by a foreign agent. As a consequence, the host becomes incapable of producing immune mediators such as the cytokines that play a crucial role in the defense against infection. Thus, the susceptibility of the host towards infection increases. Moreover, the lack of immune mediators that regulate cell proliferation, cell differentiation and cell death may result in malignant growth. For example, impaired host resistance as well as increased incidence of neoplastic disease is seen in patients treated with immunosuppressive drugs. In contrast, overstimulation of the immune system by a bacterial toxicant or a chemical may induce an excess of proinflammatory mediators which can cause death of host cells, autoimmunity due to activation of autoreactive T cells or allergic responses. Interestingly, such an overstimulation of the immune system is often accompanied by a state of hypersensitivity where detrimental synergistic effects are seen in the presence of additional immunostimulators. This can be followed by an immunosuppressed state, that facilitates the survival of invaded pathogens.

3.2 ROLE OF TUMOUR NECROSIS FACTOR α AND ITS CELLULAR SOURCES

Tumour necrosis factor α (TNFα), a proinflammatory cytokine which is produced among others by macrophages and T cells, turned out to be one of the most harmful endogenous mediators following overstimulation of the immune system. This cytokine is well known as a modulator of apoptosis in vertebrates and there are examples of both induction and suppression of apoptosis by this cytokine. However, in the context of immunotoxicology, TNFα usually induces cell death. TNFα contributes to rapid multi organ failure due to septic complications (Beutler *et al.,* 1985, Tracey *et al.,* 1987) and it is the key mediator of liver injury and lethality induced by lipopolysaccharide (LPS) (Lehmann *et al.,* 1987, Tiegs *et al.,* 1990, Leist *et al.,* 1995a) or superantigens (Miethke *et al.,* 1992, Miethke *et al.,* 1993a, Nagaki *et al.,* 1994) in mice sensitized with the hepatic transcriptional inhibitor D-galactosamine (GalN) (for further details on the role of sensitization see 3.6). TNFα also plays an important role in promoting liver injury elicited either by the T cell mitogenic plant lectin concanavalin A (ConA) (Mizuhara *et al.,* 1994, Gantner *et al.,* 1995a, Ksontini *et al.,* 1998) or by *Pseudomonas aeruginosa* exotoxin A (PEA), a bacterial toxin primarily known as an inhibitor of protein synthesis and recently described as an inducer of T cell-dependent liver injury (Schümann *et al.,* 1998). Surprisingly, TNFα even contributes

to liver failure caused by hepatotoxins such as the deathcup poison α-amanitin (Leist *et al.,* 1997a) or the environmental contaminant 2, 3, 6, 7-tetrachlorodibenzo-p-dioxin (TCDD) (Taylor *et al.,* 1992). In the latter cases, TNFα production is probably a secondary event, caused by inflammation due to TNFα-independent necrotic tissue damage and/or by translocation of LPS from the intestine. At least in the case of TCDD-induced TNFα-dependent lethality, LPS-resistant C3H/HeJ mice were much less sensitive than normal mice (Clark and Taylor, 1994). The role of TNFα in mediating the effects of hepatic nongenotoxic carcinogens is discussed in detail in Chapter 8.

Macrophages are supposed to be the source of TNFα in LPS-treated animals, since transfer of macrophages from LPS-sensitive C3H/HeN mice to LPS-resistant C3H/HeJ mice renders C3H/HeJ mice susceptible to GalN/LPS-induced lethality (Freudenberg *et al.,* 1986). In all other models mentioned above the cellular source of TNFα has not been identified until now. We have shown by means of immunofluorescent staining of liver sections that soon after PEA injection there is a massive staining of TNFα in the liver, co-localized with the resident liver macrophages, the Kupffer cells, but not with T cells (Schümann *et al.,* 1998). This is especially exciting when taking into account that TNFα is not measurable in plasma at this early stage (Schümann *et al.,* 1998). This is clear evidence for the suggestion that circulating cytokine levels only insufficiently reflect local tissue concentrations, well known as the concept of 'the tip of the iceberg' (Cavaillon *et al.,* 1992). The absence of T cells results in complete abolishment of TNFα production by Kupffer cells (Schümann *et al.,* 1998), suggesting that T cells, though not the source of TNFα, activate Kupffer cells to produce TNFα. Intravenous administration of ConA to mice induces TNFα production by Kupffer cells and $CD4^+$ T cells (G.Tiegs, unpublished results) and causes liver injury (Tiegs *et al.,* 1992) that requires the resident liver T cell population, $NK1.1^+$ T cells (Toyabe *et al.,* 1997). This cell type may have wide relevance for Kupffer cell-dependent TNFα production.

3.3 HYPERSENSITIVITY AND HYPORESPONSIVENESS

The toxicity of a xenobiotic that works by overstimulating the immune system clearly depends on the immunostatus of the intoxicated mammalian organism. This is equally applicable whether the toxicant is man-made or natural. This status is discussed in the following section.

3.3.1 Hypersensitivity

The superantigen staphylococcal enterotoxin B (SEB) that specifically stimulates T cells carrying particular V_β regions in their T cell receptors, causes a short-lasting hyperreactive state (1–2 h) of mouse lymph node cells, as assessed by a pronounced increase of

proliferation and IL-2 production after *ex vivo* restimulation with SEB (Wahl *et al.,* 1993, Miethke *et al.,* 1993b). Co-administration of SEB together with LPS induces a synergistic and lethal cytokine response syndrome in mice, whereas the same dose of each toxin given alone fails to induce lethality (Blank *et al.,* 1997). The proinflammatory cytokine interferon-γ (IFN-γ) is a key mediator of the synergism since passive immunization with anti-IFN-γ mAb prevented TNFα-dependent lethality of SEB *plus* LPS (Blank *et al.,* 1997). However, the exact role of IFN-γ remains to be elucidated. IFN-γ may have sensitized macrophages to enhanced TNFα production upon LPS stimulation. Moreover, IFN-γ may have also sensitized target cells to TNFα-induced cell death (for further details on IFN-γ-dependent sensitization of target cells see 3.6.5.1).

Another example of hyper sensitivity was observed 24 h after injection of a low dose of LPS to mice. Low dose LPS activates macrophages which in turn produce little, *per se,* untoxic amounts of cytokines, such as TNFα and IL-12. As a consequence, IL-12 induces the production of IFN-γ predominantly in NK1.1$^+$ T cells, but not in natural killer (NK) cells (Ogasawara *et al.,* 1998). In turn, IFN-γ primes macrophages and possibly other cell types. Such a priming renders mice highly susceptible to a second injection of LPS that results in massive production of proinflammatory cytokines and lethality, well known as the generalized Shwartzman reaction.

Small amounts of endotoxin also augment liver injury from a variety of hepatotoxicants including carbon tetrachloride, ethanol, halothane, and others (for review see Roth *et al.,* 1997). Vice versa, TCDD- (Rosenthal *et al.,* 1989, Clark *et al.,* 1991) and carbon tetrachloride-dependent hypersensitivity to LPS, accompanied by significantly increased levels of TNFα, have been described. These examples imply that there are fundamental similarities between the immunotoxicologic effects of natural and non-natural xenobiotics.

3.3.2 Hyporesponsiveness

As described above, a single dose of SEB causes early short-lasting hyperreactivity of lymph node cells. This state is followed by immunosuppression that is characterized by a V_β-unrestricted unresponsiveness of T cells, i.e. the inability of spleen cells to release IL-2 upon stimulation with any polyclonal T cell mitogen, also other than SEB (Muraille *et al.,* 1997). This state lasts for about 2–3 days and is followed by selective apoptosis and anergy of superantigen-responsive T cells (Rellahan *et al.,* 1990, Perkins *et al.,* 1993). The general immunosuppressed state is particularly advantageous to the superantigen-producing germ because it facilitates the survival of the invaded bacteria. On the other hand, the immunosuppressed state protects mice from toxicity of other superantigens, such as toxic shock syndrome toxin 1 (TSST-1) (Miethke *et al.,* 1993b) or the T cell-stimulatory toxin PEA (Schümann *et al.,* 1998).

A great deal of effort was made to characterize the hyporesponsive state induced by LPS. Exposure of mice to LPS, even at a very low dose, leads to a down-regulation of the cytokine response to a second high dose LPS challenge (Flohé *et al.,* 1991, Zuckerman and Evans, 1992), and renders mice resistant to GalN/LPS-induced lethality (Freudenberg and Galanos, 1988). Induction of tolerance is mediated by macrophages (Freudenberg and Galanos, 1988). Two possible explanations for LPS-induced hyporesponsiveness may be given: (1) LPS stimulates macrophages to produce anti-inflammatory cytokines, such as IL-10 and TGF-β1, which suppress the production of proinflammatory cytokines, such as TNFα, after a second exposure to LPS. Such a mechanism was suggested by *in vitro* experiments with human peripheral blood mononuclear cells (PBMC), preincubated with anti-IL-10 mAb or anti-TGF-β1 mAb, which both prevented the induction of LPS tolerance (Randow *et al.,* 1995). However, tolerance induction was still possible in IL-10-deficient mice (Berg *et al.,* 1995), pointing to additional factors, other than IL-10, that down-regulate the TNFα response to the second LPS challenge. (2) LPS-induced production of TNFα and IL-1 is responsible for the induction of target cell resistance to TNFα-induced cytotoxicity because pretreatment with LPS, TNFα, or IL-1 protected mice from GalN/TNFα-induced lethality (Libert *et al.,* 1991). Probably, both aspects play a role in LPS-mediated hyporesponsiveness of mice to a second LPS challenge.

Drugs that elevate the second messenger cyclic adenosine monophosphate (cAMP) in leukocytes, e.g. inhibitors of phosphodiesterases (PDEs), protect mice from GalN/LPS-(Fischer *et al.,* 1993, Jilg *et al.,* 1996), GalN/SEB- (Gantner *et al.,* 1997), and ConA-(Gantner *et al.,* 1997) induced liver injury. The underlying mechanism has not been clarified yet. Inhibition of NF-κB activation that participates in the transcription of the TNFα gene (Ollivier *et al.,* 1996, Yao *et al.,* 1997) or up-regulation of TNFα-suppressive cytokines such as IL-10 (Gantner *et al.,* 1997) may be involved. Indeed, plasma levels of IL-10 in mice significantly increased after GalN/LPS (Jilg *et al.,* 1996), GalN/SEB (Gantner *et al.,* 1997) or ConA (Gantner *et al.,* 1997) challenge in the presence of PDE inhibitors. Concomitantly, plasma concentrations of TNFα significantly decreased. Furthermore, endogenously produced (Florquin *et al.,* 1994a) or exogenously administered IL-10 (Bean *et al.,* 1993) has been shown to protect mice from GalN/SEB-induced TNFα-dependent liver injury. Reduced TNFα plasma levels upon PDE inhibition may also be explained by increased shedding of TNF receptors (Jilg *et al.,* 1996). This is supported by the observation that PDE inhibitors also conferred resistance to GalN/TNFα-induced liver injury (Jilg *et al.,* 1996). In this case IL-10-mediated reduction of TNFα production can be excluded as the mechanism of protection. Very recently, dibutyryl cAMP has been shown to protect mice from GalN/TNFα-induced hepatic failure, correlating with an enhanced expression of heat shock protein 70 (Hsp-70) in hepatocytes of GalN/TNFα-treated mice (Takano *et al.,* 1998) (for further details on Hsp-70 as a cytoprotective protein see 3.6.3). This finding provides evidence for an additional mechanism by which cAMP elevation might protect mice from TNFα-mediated hepatocytotoxicity.

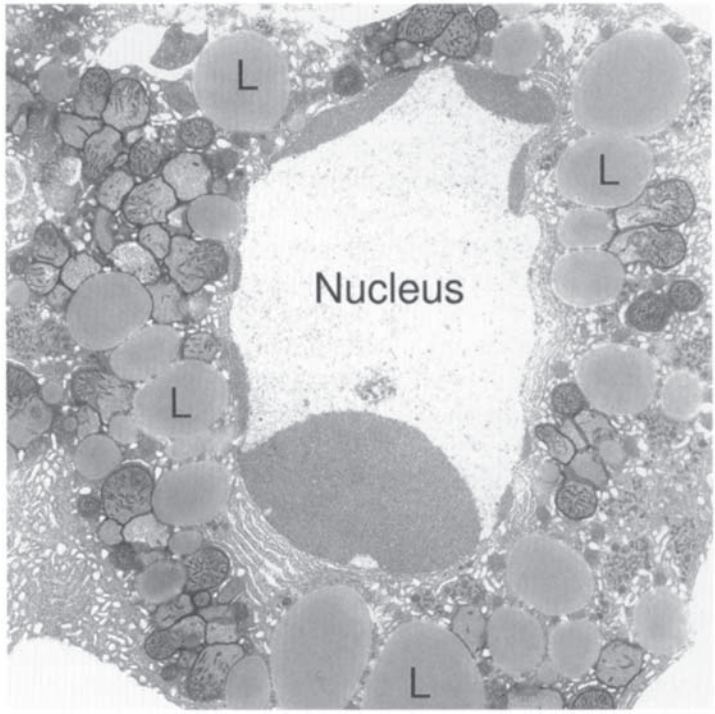

Figure 3.1 Apoptosis in immunotoxicology. In all TNFα-dependent models of experimental liver injury in mice, hepatocytes die by apoptosis. The figure shows an electron micrograph of a nucleus of an apoptotic hepatocyte in a mouse liver 5 h after treatment with GalN/TNFα, as described elsewhere (Angermüller *et al.,* 1998) (the micrograph was kindly provided by Dr S.Angermüller, Heidelberg; L=lipid droplets). In the nucleus chromatin is condensed at the nuclear wall as it is typical of apoptosis.

3.4 MECHANISMS OF CELL DEATH IN MURINE MODELS OF IMMUNE-MEDIATED TOXIC LIVER INJURY

In the TNFα-dependent models of experimental liver injury in mice, liver necrosis-associated transaminase release is preceded by hepatic internucleosomal DNA fragmentation, as quantified in liver homogenates by an ELISA specific for histone-bound DNA (Gantner *et al.,* 1995b, Leist *et al.,* 1995a, 1997a, Schümann *et al.,* 1998). Additionally, the internucleosomal character of DNA cleavage was verified by analysis of liver DNA on agarose gels (Gantner *et al.,* 1995b, Leist *et al.,* 1995a, 1997a, Schümann *et al.,* 1998). In all cases, DNA fragmentation showed the specific ladder pattern, as it is typical of apoptosis. Most importantly, microscopic analysis proved that liver cells die by apoptosis following any treatment mentioned above (see Figure 3.1), i.e. GalN/TNFα (Leist *et al.,* 1995a, Angermüller *et al.,* 1998), GalN/LPS (Leist *et al.,* 1995a), GalN/superantigen (Gantner *et al.,* 1995b); ConA (Leist *et al.,* 1995a), PEA (Schümann *et al.,* 1998), or a-amanitin

(Leist *et al.,* 1997a). This was demonstrated by detection of hepatocellular apoptotic bodies in liver tissues of treated mice.

Until now it still remains unclear why apoptotic cell death is accompanied by inflammatory necrosis at later stages. Two explanations of this phenomenon can be discussed: (1) Due to the loss of the inner mitochondrial membrane potential during apoptosis (Zamzami *et al.,* 1995), a process known as mitochondrial permeability transition (PT), excess generation of reactive oxygen intermediates involved in apoptotic signalling (Hockenberry *et al.,* 1993, Kane *et al.,* 1993, Sandstrom *et al.,* 1994, Zamzami *et al.,* 1995), may cause oxidative stress leading to necrosis. Additionally, loss of ATP due to PT may prevent the energy-dependent process of apoptosis, thereby inevitably changing the apoptotic signal cascade into a necrotic one (Leist *et al.,* 1997b) (2). As evidenced by electron microscopy, it appears that phagocytosis of an increasing amount of apoptotic bodies overtaxes the capacity of the phagocytizing neighbouring hepatocytes, thereby leading to the burst of lysosomes, which in turn causes lytic cell death (S.Angermüller, personal communication).

3.5 ROLE OF CASPASES

A very recent work demonstrated a double-edged role of cysteine aspartyl proteases, called caspases (for further details on caspases see Chapter 2), in the scenario of cell death (Vercammen *et al.,* 1998). Primarily, caspases are known as the executioners of apoptosis. Peptide inhibitors of caspases protect cells from apoptosis (for review see Villa *et al.,* 1997). Interestingly, it has been shown that the general tripeptidic caspase inhibitor Z-VAD.fmk protects Jurkat cells not only from apoptosis but also from necrosis triggered by activation of the CD95/Fas receptor molecule (Leist *et al.,* 1997b), a member of the TNF receptor superfamily. However, a novel role of caspases has recently been described, i.e. prevention of necrosis (Vercammen *et al.,* 1998). TNFα-mediated necrosis of murine L929 fibrosarcoma cells was enhanced in the presence of the broad spectrum caspase inhibitors ZVAD.fmk and Z-D.fmk as well as of the caspase-1-like enzyme inhibitor Ac-YVAD.cmk and the caspase-3-like enzyme inhibitor Z-DEVD.fmk (Vercammen *et al.,* 1998). Caspases seem to downmodulate necrotic pathways in these cells by inhibiting TNFα-induced oxygen radical production. This was concluded from the fact that the oxygen radical scavenger butylated hydroxyanisole inhibited Z-VAD.fmk-dependent sensitization to TNFα-induced cell necrosis (Vercammen *et al.,* 1998). Until now, no *in vivo* data has been available to confirm the hypothesis of the role of caspases in preventing necrosis. In all studies dealing with the influence of caspase inhibitors on TNFa-mediated or agonistic anti-Fas-antibody-triggered liver injury in mice, the inhibitors tested, i.e. Z-VAD.fmk (GalN/TNFα (Künstle *et al.,* 1997), GalN/LPS (Jaeschke *et al.,* 1998) and anti-Fas (Rodriguez *et al.,* 1996, Künstle *et al.,* 1997)), Ac-YVAD.cmk (GalN/TNFα (Schümann and Tiegs, unpublished results) and anti-Fas (Rodriguez *et al.,* 1996, Rouquet

et al., 1996)), and Ac-DEVD.CHO (anti-Fas (Suzuki, 1998)), showed protective properties. These protective effects include prevention of apoptosis, necrosis, and death. Furthermore, Z-VAD.fmk also protected mice from PEA-induced apoptotic and necrotic liver damage (Schümann *et al.,* 1998), and caspase-1-deficient mice were resistant to ConA-induced hepatitis (Ksontini *et al.,* 1998). Hence, *in vivo* caspase inhibition failed to enhance necrotic cell damage that usually follows hepatocellular apoptosis in the models of liver injury.

3.6 TARGET CELL SENSITIZATION TO TNFα-MEDIATED CYTOTOXICITY AND SELF-PROTECTION

Despite the TNFα-dependence of liver injury observed in the several models mentioned above, sole administration of a reasonable TNFα dose neither induces apoptosis nor necrosis in mice (Tiegs *et al.,* 1989, Leist *et al.,* 1995a). Hence, TNFα is unable to cause cell death in the absence of additional factors that sensitize target cells. A common sensitizing mechanism of liver parenchymal cells to TNFα-induced apoptosis and necrosis is the transcriptional inhibition by the uracil nucleotide depleting aminosugar GalN (Galanos *et al.,* 1979, Tiegs *et al.,* 1989, 1990) or by the cytostatic drug actinomycin D (ActD) (Leist *et al.,* 1994, 1995a). The mechanism by which transcriptional inhibition provides susceptibility of hepatocytes to TNFα is unresolved. One possible explanation may be given in terms of inhibition of the expression of so-called 'protective proteins'. Activation of the transcription factor 'nuclear factor-κB' (NF-κB) by TNFα is a crucial step in the TNFα-induced self-protection from TNFα-induced cell death. The functional role of the 'c-jun N-terminal kinase' (JNK) which is also activated by TNFα is still under debate (Liu *et al.,* 1996, Lee *et al.,* 1997, Yeh *et al.,* 1997). However, if activation of NF-κB is prevented, TNFα-induced cell death occurs without additional sensitization in different cell types (Beg and Baltimore, 1996, Liu *et al.,* 1996, van Antwerp *et al.,* 1996, Wang *et al.,* 1996). The intracellular signalling cascade that leads to the activation of NF-κB is not fully understood. The presence of functional 'TNF receptor associated factor 2' (TRAF-2), that intracellularly associates with TNF receptor 1 as well as TNF receptor 2 (for further details on the TNF receptors see 3.7) upon TNF receptor activation (Rothe *et al.,* 1994, Hsu *et al.,* 1996a, b), has been shown to protect cells from TNFα-induced cell death (Lee *et al.,* 1997, Yeh *et al.,* 1997). It has been assumed that TRAF-2 stimulates the activation of NF-κB, because cells expressing a dominant-negative TRAF-2 molecule were unable to activate NF-κB upon triggering of TNF receptor 1 (Hsu *et al.,* 1996a) or TNF receptor 2 (Rothe *et al.,* 1995a). On the other hand, experiments with cells from TRAF-2-deficient mice (Yeh *et al.,* 1997) and from mice overexpressing a dominant negative form of the TRAF-2 protein in lymphocytes (Lee *et al.,* 1997) have shown that TNFα-induced NF-κB activation is still possible. However, NF-κB activation was delayed in case of TRAF-2-deficiency (Yeh

et al., 1997) and possibly compensated by co-existing wild-type TRAF-2 protein in the transgenic mice (Lee *et al.,* 1997). Hence, recruitment of TRAF-2 and activation of NF-κB are possibly partially independent events that both provide the TNFα-induced protection from TNFα-induced cell death.

A very recent publication showed a participation of reactive oxygen intermediates in the activation of NF-κB and the apoptotic protease caspase-3 by means of overexpression of the TNFα-inducible manganese superoxide dismutase (Mn-SOD) in human breast cancer MCF-7 cells. Overexpression of Mn-SOD prevented the activation of both NF-κB and caspase-3 by TNFα (Manna *et al.,* 1998). *In vivo,* TNFα-dependent activation of NF-κB has been observed in livers of ConA-injected mice (Trautwein *et al.,* 1998a). Drug-induced activation of NF-κB correlated with protection from ConA-induced liver injury (Tiegs *et al.,* 1998). The whole set of cytoprotective proteins induced by NF-κB is unknown, but there are some 'hot' candidates, among which are the cellular inhibitor of apoptosis protein 2 (c-IAP-2), the zinc ringer protein A20, the heat shock protein 70 (Hsp-70), and the above mentioned manganese superoxide dismutase (Mn-SOD) whose inhibition by antisense-MnSOD-RNA rendered 293 human embryonic kidney cells sensitive to TNFα (Wong *et al.,* 1989). Furthermore, pretreatment of rats with TNFα prevented liver damage caused by carbon tetrachloride. This was associated with an increased activity of Mn-SOD in the liver (Sato *et al.,* 1995), thus pointing to a very central role of cytoprotective proteins in the toxicity of both, natural and non-natural xenobiotics.

3.6.1 Cellular inhibitor of apoptosis protein 2 (c-IAP-2)

Overexpression of the NF-κB-dependent c-IAP-2, a protein of the IAP family that associates with TRAF-2 (Rothe *et al.,* 1995b), has been shown to protect HeLa cells from TNFa-induced apoptosis and to contribute to the activation of NF-κB (Chu *et al.,* 1997). Cells overexpressing a mutated, not inactivatable form of the NF-κB-inhibitory I-κB protein loose their c-IAP-2-conferred resistance to TNFα-induced apoptosis (Chu *et al.,* 1997). Furthermore, c-IAP-2 seems to directly inhibit caspase-3-like enzymes, and overexpression of c-IAP-2 has been shown to inhibit etoposide- or Fas-triggered activation of caspase-3-like enzymes and apoptosis in human 293 cells (Roy *et al.,* 1997). Taken together, NF-κB-dependent expression of c-IAP-2 may confer resistance to apoptosis triggered by various stimuli. This may be achieved by enhancing the activation of NF-κB *and* by the inhibition of caspase-3-like enzymes which are regarded as central executioners of many apoptotic pathways.

3.6.2 The A20 protein

Although capable of inhibiting TNFα-induced apoptosis (Opipari *et al.,* 1992, Jäättelä *et al.,* 1996, Ferran *et al.,* 1998), the NF-κB-dependent (Krikos *et al.,* 1992), TNFα-inducible

(Dixit *et al.,* 1990, Opipari *et al.,* 1992) A20 protein inhibits further activation of NF-κB (Song *et al.,* 1996, Jäättelä *et al.,* 1996, Cooper *et al.,* 1996). Inhibition of NF-κB activation by A20 may therefore be a mechanism of down-regulating (e.g. TNFα-induced) NF-κB activation. The mechanism by which A20 inhibits NF-κB activation and apoptosis remains unknown. Hence, A20 may provide a very interesting example of a molecule that possibly attenuates inflammation. The synthesis of proinflammatory cytokines, such as TNFα and IFN-γ, depends on the activation of NF-κB, while at the same time A20 has a negative effect on TNFα-induced apoptosis.

3.6.3 Heat shock protein 70 (Hsp-70)

Induction of Hsp-70 following exposure to TNFα has been observed in cardiac myocytes (Nakano *et al.,* 1996), hepatocytes (G.Tiegs, unpublished results), and other cell types. An increase in Hsp-70 protein also occurs after incubation of rat hepatocytes with IFN-γ *plus* IL-1β (Kim *et al.,* 1997). In the latter case, accumulation of Hsp-70 turned out to depend on the formation of nitric oxide (NO). This was concluded from the fact that the NO synthase inhibitor N^G-monomethyl-L-arginine (NMMA) prevented the cytokine-dependent accumulation of Hsp-70 (Kim *et al.,* 1997). Accordingly, treatment of rat hepatocytes with the NO donor S-nitroso-N-acetylpenicillamine (SNAP) induced the expression of Hsp-70, and furthermore protected the cells from apoptosis triggered by TNFα plus an inhibitor of transcription (Kim *et al.,* 1997). The functional role of Hsp-70 as a cytoprotective component has been proven by an antisense oligomer to Hsp-70 that blocked Hsp-70 expression and prevented the NO-dependent protection from TNFα-induced apoptosis of sensitized cells (Kim *et al.,* 1997). The combination of IFN-γ and IL-1β is a well known inducer of the NF-κB-dependent inducible NO synthase (Geller *et al.,* 1993, Xie *et al.,* 1994). Hence, TNFα may also cause an increase of Hsp-70 via NF-κB-dependent induction of the inducible NO synthase, thereby protecting the exposed cell from TNFα and possibly other apoptotic stimuli. This hypothesis is supported by a life-saving role of TNFα pretreatment (Libert *et al.,* 1991) and a hepatoprotective effect of NO donor pretreatment (Bohlinger *et al.,* 1995a,b) in the *in vivo* model of GalN/TNFα-induced liver injury, although a causal relationship between TNFα pretreatment, NO synthesis and induction of Hsp-70 has not yet been proven. A possible protective role of Hsp-70 induction *in vivo* is evidenced by a correlation of dibutyryl cAMP-elicited Hsp-70 expression and protection from GalN/TNFα-induced liver injury (Takano *et al.,* 1998).

3.6.4 The role of a proliferation signal induced by TNFα

It has been demonstrated that TNFα not only protects cells from its own deleterious effects, but also induces proliferation of hepatocytes via activation of TNF receptor 1. This has been demonstrated by an impaired mitogenic response of hepatocytes during CCl_4-induced liver

injury in TNF receptor 1-deficient mice (Yamada and Fausto, 1998). The impaired mitogenic response correlated with reduced DNA binding of the transcription factors NF-κB and 'signal transducer and activator of transcription 3' (STAT3), as well as with lower plasma levels of IL-6 (Yamada and Fausto, 1998). These components may be important for the TNFα-dependent hepatocyte mitogenic response contributing to liver regeneration that counteracts apoptosis. Mechanistic studies on liver regeneration have been performed in the model of ConA-induced hepatitis in mice. They revealed that the nuclear expression of a protein responsible for cell cycle arrest at the G1/S checkpoint, but presumably necessary for G0/G1 shift, i.e. CCAAT/enhancer-binding protein-β/liver-enriched activating protein (C/EBP-β/LAP), is induced soon after ConA injection as a consequence of TNFα production, since passive immunization with anti-TNFα Ab prevented expression of C/EBP-β/LAP (Trautwein *et al.,* 1998b). The concentration of C/EBP-β/LAP returned to normal levels 24 h after ConA administration, and the hepatocytes entered the S-phase of the cell cycle (Trautwein *et al.,* 1998b). This example shows that cell cycle and apoptosis are contrarily regulated. A mediator possibly responsible for the switch from cell destruction to cell proliferation is IL-6. This cytokine is produced in a TNFα-dependent manner via activation of TNF receptor 1 upon partial hepatectomy in mice, and its lack in TNF receptor 1-deficient mice correlates with an impaired nuclear translocation of STAT3 and impaired DNA synthesis (Yamada *et al.,* 1997). DNA binding of STAT3 is also prevented in IL-6-deficient mice after partial hepatectomy (Cressman *et al.,* 1996). Taken together, one might hypothesize that TNFα induces C/EBP-p/LAP and IL-6, IL-6 in turn induces nuclear translocation of STAT3, and STAT3 contributes to cell proliferation and possibly also provides yet unknown anti-apoptotic signals, shifting the situation from cell destruction to cell survival and proliferation, which results in liver regeneration. This hypothesis is supported by the finding that mice pretreated with IL-6 were resistant to ConA-induced liver injury (Mizuhara *et al.,* 1994). Furthermore, knockout mice lacking the functional Rel A component of NF-κB have a defect in normal liver development (Bellas *et al.,* 1997). Since NF-κB is associated with cell cycle progression in hepatocytes during liver regeneration, NF-κB also seems to contribute to enhanced proliferation of hepatocytes. It may be assumed that the sequence of events for the initiation of liver regeneration is: TNFα→TNF receptor 1→NF-κB→IL-6→STAT3→DNA synthesis (Yamada *et al.,* 1997).

3.6.5 Cryptic sensitizations and the role of additional mediators

PEA, in contrast to LPS or superantigen, is able to induce TNFα-mediated hepatotoxicity without further sensitization (Schümann *et al.,* 1998). This could be easily explained by the immanent protein synthesis inhibitory property of PEA (Pavlovskis and Shackelford, 1974, Iglewski and Kabat, 1975) which mono-ADP-ribosylates eukaryotic translation elongation factor 2 (eEF-2) (Iglewski *et al.,* 1977), thus providing a similar sensitization as shown by

GalN or ActD. This example shows that a naturally occurring toxin, such as PEA, can possess different properties, i.e. in this case immunostimulation and protein synthesis inhibition, that contribute to its toxicity. TNFα is not the only mediator of PEA-induced liver injury, since perforin-deficient mice showed a significantly reduced susceptibility to PEA (Schümann *et al.,* 1998). Hence, PEA is an excellent example of a toxin that evokes multiple immunotoxic reactions. In particular, TNFα might preactivate antigen presenting cells such as dendritic cells thereby facilitating the activation of cytotoxic T cells (Bennett *et al.,* 1998, Ridge *et al.,* 1998, Schoenberger *et al.,* 1998) that in turn produce cytotoxic mediators like perforin.

3.6.5.1 The dual role of interferon-γ

ConA induces liver injury independently of sensitization (Tiegs *et al.,* 1992). ConA neither inhibits transcription nor translation (Leist *et al.,* 1995a), as do GalN, ActD, or PEA. The mechanisms by which ConA sensitizes the mouse liver to TNFα are unknown. In contrast to the GalN models, passive immunization with anti-IFN-γ Ab protected mice from ConA-induced liver failure (Küsters *et al.,* 1996). IFN-γ is known to sensitize p53-negative human colon adenocarcinoma cells (HT-29) and other cell lines to different apoptotic stimuli, including TNFα and agonistic Fas Ab (Fransen *et al.,* 1986, Ossina *et al.,* 1997). IFN-γ also sensitizes mice to TNFα *in vivo* and it enhances the effects of very high doses of LPS or TNFα (Doherty *et al.,* 1992). Following incubation with IFN-γ, a lot of potentially apoptosis-promoting proteins accumulate: TNF receptor 1 (Tsujimoto *et al.,* 1986, Ossina *et al.,* 1997), Fas (Ossina *et al.,* 1997), several caspases (Tamura *et al.,* 1996, Chin *et al.,* 1997, Ossina *et al.,* 1997), and Bak, a pro-apoptotic member of the Bcl-2 protein family (Ossina *et al.,* 1997). p53-positive cells, e.g. primary cultured hepatocytes, react with enhanced expression of the tumoursuppressor and apoptosis-mediator p53 in response to IFN-γ (Kano *et al.,* 1997). IFN-γ also induces the expression of the 2'-5' oligoadenylate (2–5A) synthetase. Its product, 2–5A, activates the rRNA degrading RNase L (Johnston and Torrence, 1984), thereby indirectly inhibiting protein synthesis. Cells from RNase L-knockout mice are less susceptible to different apoptotic stimuli, including TNFα (Zhou *et al.,* 1997). In addition to the 2–5A synthetase, IFN-γ induces the expression of the 'double-stranded RNA-dependent protein kinase' (PKR), a serine/threonine kinase that phosphorylates eukaryotic translation initiation factor 2 (eIF-2), thereby inhibiting protein biosynthesis. Expression of a dominant-negative, catalytically inactive PKR mutant protected NIH3T3 cells from apoptosis induced by different stimuli, including TNFα (Srivastava *et al.,* 1998), and overexpression of a nonphosphorylatable eIF-2 partially protected cells from TNFα-induced apoptosis (Srivastava *et al.,* 1998). The so-called 'death associated protein kinase; (DAP kinase) has also been implicated in the IFN-γ-increased sensitivity of cells to apoptotic stimuli. The DAP kinase is induced by IFN-γ (Deiss *et al.,* 1995). DAP kinase overexpressing tumour cells displayed higher sensitivity to the apoptotic effects of TNFα (Inbal *et al.,* 1997), and a catalytically inactive DAP kinase mutant, displaying dominant-negative features, protected IFN-γ-sensitive HeLa cells from

IFN-γ-induced cell death (Cohen *et al.,* 1997). IFN-γ does not only sensitize cells to apoptosis, but also inhibits cell proliferation via activation of STAT1. STAT1-deficient human USA cells show IFN-γ-induced growth inhibition only when they express complete STAT1 activity (Bromberg *et al.,* 1996). Taken together, there exist many possible mechanisms by which IFN-γ may contribute to ConA-induced TNFα-mediated hepatocellular death *in vivo*.

IFN-γ, however, is not necessarily an enhancer of TNFα toxicity. Under appropriate circumstances it can also slow down cytotoxic processes, probably by induction of the inducible NO synthase and production of NO. Mice deficient in the IFN-γ receptor a chain are significantly more sensitive to the administration of agonistic anti-CD3 T cell activating antibodies. These mice, in contrast to wild-type mice, die as a consequence of T cell activation (Matthys *et al.,* 1995). The higher susceptibility of the IFN-γ receptor knockout mice to anti-CD3-induced death is associated with an impaired NO production, and wild-type mice can be sensitized to anti-CD3 treatment by the co-administration of an inhibitor of NO synthase (Matthys *et al.,* 1995). We have shown that passive immunization with anti-IFN-γ Ab enhances liver injury after treatment of mice with PEA (Schümann and Tiegs, unpublished results). These findings are in contrast to the results obtained with ConA-treated mice (Küsters *et al.,* 1996). A protective role of IFN-γ-dependent NO synthesis was also observed in mice challenged with SEB. Antibodies to IFN-γ inhibited SEB-induced NO formation (Florquin *et al.,* 1994b). An otherwise non-lethal SEB dose turned out to be lethal when co-injected with an inhibitor of NO synthase (N-nitroso-L-arginine methyl ester (L-NAME)) (Florquin *et al.,* 1994b). L-NAME/SEB-induced lethality was accompanied by enhanced synthesis of TNFα and IFN-γ. TNF as well as IFN-γ contributed to death as shown by the protective effect of passive immunization with anti-TNF or anti-IFN-γ Ab (Florquin *et al.,* 1994b).

In summary, it becomes clear that IFN-γ is able to play distinct roles in the process of intoxication. Toxicity enhancing as well as toxicity decreasing effects have been described. The role of IFN-γ in immunotoxicology can therefore not be clearly defined and has to be tested in each individual case.

3.6.5.2 The role of Fas

One of the most prominent cell death-mediating receptors is Fas (=CD95). Fas is constitutively expressed in a wide range of tissues, including the liver (for review see Galle and Krammer, 1998). The Fas ligand (FasL) is predominantly expressed in T cells, but can also be induced in other cell types. Administration of an agonistic anti-Fas antibody in mice results in rapid liver failure and death of the animals (Ogasawara *et al.,* 1993). Furthermore, Fas-mediated target cell killing has also been implicated in a murine hepatitis B model: transgenic mice, constitutively expressing hepatitis B virus (HBV) envelope proteins in hepatocytes, develop severe acute necroinflammatory liver disease after injection of an MHC-restricted $CD8^+$ CTL clone recognizing the HBV surface antigen.

Liver injury and death were prevented by a soluble Fas-Fc construct (Kondo *et al.,* 1997). Immunohistology of liver tissue from patients with chronic HBV infection showed highly elevated Fas expression on hepatocytes compared to liver tissue from healthy individuals (Galle *et al.,* 1995). Hence, Fas-mediated target cell killing might also be involved in one of the described experimental models of immunotoxicologically mediated liver injury.

Many functional studies have been performed using Fas-mutated *lpr/lpr* mice. These mice differ from wild-type mice in many aspects. It has been demonstrated, for example, that peritoneal macrophages from MRL *lpr/lpr* mice overproduce inflammatory cytokines, including TNFα, upon stimulation with SEB (Edwards III. *et al.,* 1996). This is possibly a result of impaired Fas-mediated apoptosis. Therefore, experimental results concerning the role of Fas in target cell killing, obtained from *lpr/lpr* mice, have to be treated with caution. Better tools for investigation of the role of Fas in target cell killing are Fas or FasL neutralizing molecules. For example, monoclonal antibodies neutralizing the murine Fas ligand, reduced transaminase release after treatment of mice with ConA (Seino *et al.,* 1997).

There is also another aspect to the cytotoxic potential of FasL/Fas interactions in immunotoxicologically mediated organ damage. Blockade of FasL with a soluble Fas immunoadhesin failed to prevent liver injury in mice treated with ConA (in contrast to the above described results of Seino *et al.,* 1997). However, when co-administered with an inhibitor of matrix metalloproteinases, the latter being responsible for the shedding of the transmembrane FasL (Tanaka *et al.,* 1996), soluble Fas immunoadhesin reduced the metalloproteinase inhibitor-enhanced ConA-induced liver injury (Ksontini *et al.,* 1998). It has been also demonstrated by others that only the membrane-bound form, but not the soluble form, of FasL has the capacity to kill target cells (Schneider *et al.,* 1998, Tanaka *et al.,* 1998), and that soluble FasL even prevents cytotoxicity of transmembrane FasL (Tanaka *et al.,* 1998). Hence, the impact of FasL-Fas interactions on immunologically mediated toxicity seems to depend on the degree of FasL shedding.

3.7 ROLES OF THE TWO TNFα RECEPTORS

The effects of TNF are mediated by two different receptors. Both are expressed in almost all cells: TNF receptor 1 (TNFR-1), also known as p55, and the p75 TNF receptor 2 (TNFR-2), (for review see Smith *et al.,* 1994). Additionally, two forms of TNF have to be distinguished, i.e. the 26 kDa transmembrane protein (mTNF) and the 17 kDa soluble cytokine (sTNF), the latter originating from the membrane-expressed form through proteolytic cleavage by a matrix metalloproteinase (Gearing *et al.,* 1994, McGeehan *et al.,* 1994). Both forms of TNF are biologically active, and bind to the TNF receptors with different affinities. Under physiological conditions, sTNF preferentially binds to TNFR-1 (K_d=1.9×10^{-11} M *vs*. K_d=4.2×10^{-10} M of TNFR-2) (Grell *et al.,* 1998a), while the transmembrane form of TNF preferentially binds to TNFR-2 (Grell *et al.,* 1995). Both receptors are capable of independent signalling, but the signalling pathways also interfere.

Mice deficient in TNFR-1 are protected from GalN/TNF-induced hepatocyte death, and wild-type BALB/c mice, injected with human TNFα, that only activates murine TNFR-1 but not TNFR-2, develop severe liver injury (Leist *et al.,* 1995b). Furthermore, TNFR-1-deficient mice are clearly resistant to GalN/LPS-induced liver failure (Pfeffer *et al.,* 1993) and lethality (Pfeffer *et al.,* 1993, Rothe *et al.,* 1993) as well as to GalN/SEB-induced lethality (Pfeffer *et al.,* 1993). These results demonstrate the central role of TNFR-1 in mediating cell death. TNFR-1-deficient mice are also resistant to ConA- (Küsters *et al.,* 1997) or acute PEA-induced liver injury (Schümann *et al.,* 1998). However, TNFR-1 is not only disadvantageous. For example, it plays an important role in the defense of the facultative intracellular bacterium *Listeria monocytogenes* (Pfeffer *et al.,* 1993, Rothe *et al.,* 1993).

Until now, little is known about the biological role of TNFR-2. Recently, Grell *et al.* have shown that TNFα-dependent thymocyte proliferation and secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) still occurs in TNFR-1-deficient mice (Grell *et al.,* 1998b). Thus, functional TNFR-2 signalling can occur independently of TNFR1. Lucas *et al.* have revealed an important role of the mTNF-triggered TNFR-2 in the upregulation of the intercellular adhesion molecule-1 (ICAM-1) during T cell-dependent experimental cerebral malaria (Lucas *et al.,* 1997). TNFR-2-deficient mice, in contrast to wild-type mice, failed to up-regulate ICAM-1 following injection of *Plasmodium berghei* ANKA-parasitized erythrocytes, leukocytes did not infiltrate into brain tissue, and mice were protected from cerebral malaria (Lucas *et al.,* 1997). However, this is not a general mechanism, since up-regulation of ICAM-1 within the liver after injection of recombinant TNF to mice only depended on the TNFR-1 (G.Tiegs, unpublished results). ICAM-1 has been implicated in the pathogenesis of ConA-induced liver injury (Watanabe *et al.,* 1996). Thus, prevention of ICAM-1 expression, besides suppression of cell death triggering, could be one reason for the resistance of TNFR-1-deficient mice to ConA.

However, TNFR-2-deficient mice exhibit decreased sensitivity to high dose TNFα-induced lethality (Erickson *et al.,* 1994), and they are protected from acute liver injury following intoxication with ConA (Küsters *et al.,* 1997) or PEA (Schümann *et al.,* 1998). The importance of the TNFR-2 for cytotoxicity in the ConA model was supported by the fact that transgenic mice, only expressing mTNF, but not sTNF, were still susceptible to ConA-induced liver failure, even though liver damage was less pronounced than in wild-type mice (Küsters *et al.,* 1997). This result shows (1) that mere expression of the prime TNFR-2 ligand, i.e. mTNF, can mediate hepatotoxicity, and (2) that the coexistence of both TNF forms, i.e. mTNF and sTNF, is necessary for full toxicity. However, in the transgenic mice expressing only mTNF, plasma IFN-γ was significantly elevated after ConA treatment (Küsters *et al.,* 1997). Therefore, it can not be excluded that the apparent susceptibility of these mice to ConA is a result of IFN-γ overload. The role of TNFR-2 in this model was further supported by an enhanced susceptibility of transgenic mice, overexpressing human TNFR-2 in addition to their own TNF receptors, to ConA (Küsters *et al.,* 1997). Since the human TNFR-2 is widely expressed in these mice, including lymphoid and hepatic tissue,

and since these transgenic mice again showed elevated plasma IFN-γ (Küsters *et al.,* 1997), the exact functional site(s) of mTNF-TNFR-2 interaction in ConA-hepatitis have not yet been defined. However, with respect to the expression of both TNF receptors by the target cells, i.e. the hepatocytes, a functional role of both receptors for either ConA- or PEA-induced hepatocellular apoptosis *in vivo* should be taken into account.

The participation of both TNF receptors in target cell toxicity can be explained by complex interactions between the signalling pathways of TNFR-1 and -2 (for review on TNF receptor signalling see Darnay and Aggarwal, 1997). TNFR-1-signalling includes the activation of the caspase cascade as well as of the transcription factor NF-κB that protects cells from TNFα-induced cell death. TNFR-1 possesses an intracellular so-called 'death domain', comprising the binding site for the TNF receptor associated death domain protein' (TRADD), which in turn binds the caspase-8 recruiting 'Fas associated death domain protein' (FADD) as well as the NF-κB-supporting cytoprotective protein TRAF-2. TNFR-2 binds TRAF-2 directly. A recent publication suggests a mechanism by which TNF receptor 2 could support apoptosis-directed TNFR-1-signalling (Duckett and Thompson, 1997). Human 293 cells were co-transfected with TRAF-2 and a construct encoding a fusion protein consisting of the extracellular and transmembrane parts of CD28 and the intracellular part of TNFR-2. This construct has been shown to form homodimers thereby being activated without additional ligands. Interestingly, in these cells, TRAF-2 was strongly degraded, and activation of NF-κB was prevented (Duckett and Thompson, 1997). Thus, TNFR-2 may contribute to TNFR-1-mediated cell death by sensitizing cells through degradation of TRAF-2, thereby preventing activation of NF-κB.

3.8 SUMMARY

Certain microbial or chemical toxicants affect the immune system. They are able to cause acute or chronic disease by means of suppression or overstimulation of the immune system. Immunosuppression may be caused by anergy and apoptosis of immune cells while overstimulation of the immune system may result in apoptosis and secondary necrosis of target cells. One central mediator of such a target cell death is TNFα. Additional mediators or target cell sensitization are necessary in most cases to facilitate TNFα cytotoxicity. Target cell death is modulated on several levels of the intoxication pathway (see Figure 3.2). It depends on (1) the immunostatus, (2) the co-exposure to synergizing or sensitizing agents, and (3) the current equipment of the target cells with cytoprotective proteins. Furthermore, the current cell cycle phase may influence the cell's susceptibility to apoptogenic mediators. The cytoprotective proteins might be of great significance for attenuation of the toxicity of xenobiotics. The investigation of novel drugs that induce cytoprotective proteins could possibly provide new approaches to the treatment of organ damage due to intoxications with immune-active natural or chemical toxicants.

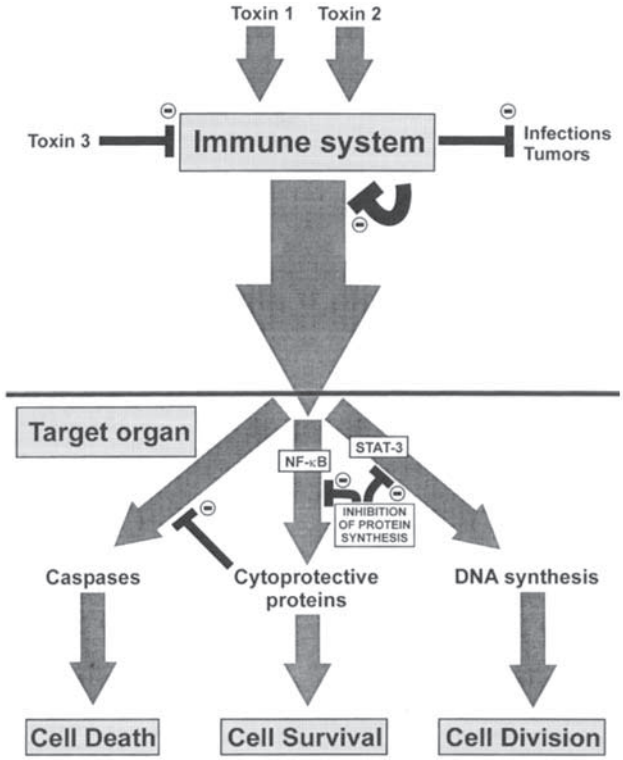

Figure 3.2 Modulation of apoptosis in immunotoxicology. The immune system can be affected by toxins (toxins 1, 2, and 3). The toxins 1 and 2 symbolize xenobiotics that overstimulate the immune system, and act together in a synergistic manner. Toxin 3, in contrast, is an immunosuppressive toxin or drug that prevents the immune system from controlling infections and tumours. Overstimulation of the immune system is characterized by the production of various proinflammatory immune mediators, most prominently TNFα, IL-1, IL-6, and IFN-γ, that affect certain target cells. The immune reaction is controlled by co-released anti-inflammatory mediators such as IL-10 or TGF-β1. In the target cells, the effector mediators elicit three different pathways that might interfere with each other. (1) The activation of caspases leads to apoptosis, (2) the NF-κB-dependent induction of cytoprotective proteins disrupts the apoptotic pathway thereby facilitating cell survival, and (3) activation of STAT 3 and possibly other transcription factors promotes the cell cycle thereby leading to cell proliferation and possibly inhibition of cell death. Inhibition of protein synthesis represents a mechanism of target cell sensitization, which probably works by inhibiting the induction cytoprotective and cell cycle proteins. The overall balance of the apoptotic, cytoprotective, and proliferative pathways in the target cell should determine the immune-mediated toxicity of a xenobiotic.

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CHAPTER 4

Apoptosis in Male Reproductive Toxicology

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4.1 INTRODUCTION

The control of spermatogenesis is extremely complex requiring testosterone from the Leydig cells situated within the interstitium and the supportive role of the Sertoli cells within the seminiferous tubules (reviewed in Sharpe, 1994). During spermatogenesis, spermatogonia divide by mitosis and meiosis producing haploid spermatids which then transform into spermatozoa (Figure 4.1). The synchronous development of a clone of spermatogonia should give rise to 4096 spermatids. However the actual numbers fall far short of this, since the average clone contains only about 100 cells (Russel *et al.,* 1990); cell division and cell death are both implicated in controlling the final number of spermatozoa. Industrialization and the use of chemicals for environmental and therapeutic reasons has exposed the male reproductive system to a wide variety of compounds that might pose a threat to male fertility. There is much debate as to whether sperm counts in men have declined in the last 50 years (Carlsen *et al.,* 1992, Bromwich *et al.,* 1994, Ashby *et al.,* 1997, Seino *et al.,* 1997). It is clear that there are a number of sites within the testis and a number of targets in spermatogenesis where toxicants might act (Seino *et al.,* 1997). Many environmental and 'therapeutic' toxicants have been shown to disrupt spermatogenesis

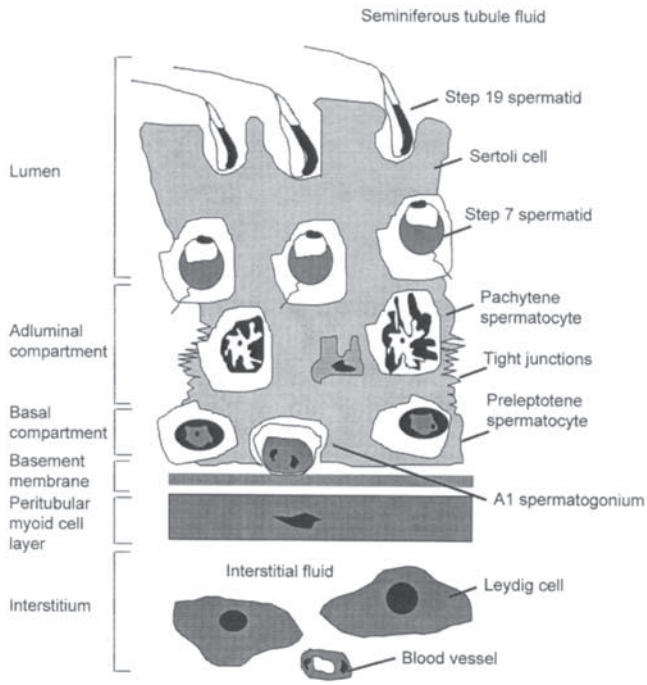

Figure 4.1 Diagram of a Sertoli cell showing its association with germ cells in the rat testes.

in rodent models. Increasingly, many of them are being shown to induce apoptosis in the testis although in most cases, the molecular mechanisms are not yet defined. This chapter will review current knowledge on the physiological role and genetic control of apoptosis in the testis, then consider toxicants that interfere with this physiology to modulate apoptosis.

4.2 ROLE OF SERTOLI AND LEYDIG CELLS

The Sertoli cell is a somatic cell within the seminiferous epithelium of the testis (Figure 4.1). This cell does not divide and, in general, is not susceptible to toxins although some industrial chemicals are known to pose a threat. The Sertoli cell provides the necessary biomolecules for the growth and development of germ cells and structural support for the germ cells so that they can maintain their cellular associations. In addition, tight junctions between the Sertoli cells serve to exclude potentially harmful substances such as toxins and antibodies thus rendering the seminiferous epithelium 'an immune privileged site'. The integrity of this cell is essential for successful spermatogenesis. Reports of Sertoli cell apoptosis are few and far between although Allan *et al.* (1988) claim that 5 Gy irradiation induces death of Sertoli cells by apoptosis in 4-day-old rats. However, when Sertoli cells are cultured, a basement membrane of laminin or matrigel is required for Sertoli cell survival. Sertoli cells cultured on plastic die by apoptosis even in the presence of known regulators of Sertoli cell function (Dirami *et al.,* 1995).

One function of Sertoli cells is to engulf and degrade dead germ cells. This phagocytosis is impaired when liposomes containing acidic but not neutral phospholipids are present *in vitro* (Shiratsuchi *et al.,* 1997). This observation along with the fact that spermatogenic cells expose phosphatidylserine on their surface when maintained in culture would suggest that these acidic phospholipids translocate from the inner to the outer leaflet of the plasma membrane during apoptosis and serve as a signal for phagocytosis by Sertoli cells. Cyclic protein-2 (CP-2) or cathepsin is expressed in the Sertoli cells and is thought to facilitate the degradation of dead germ cells once they are engulfed (Kim and Wright, 1997). The expression of CP-2 increases during testicular maturation and levels are high in all tubules that lose germ cells by apoptosis.

The Leydig cell synthesizes and secretes testosterone thus maintaining the somatic and testicular aspects of male fertility. Like Sertoli cells, somatic Leydig cells do not divide and apoptosis of Leydig cells has not been reported in the normal adult rat testis (Taylor *et al.,* 1998). Toxicants usually exert their effects on Leydig cells by interfering with steroidogenic enzymes and the action of such toxicants is often characterized by their ability to reduce peripheral androgen levels. In addition, toxicants may have indirect effects on Leydig cells perhaps by affecting the neuroendocrine axis at the hypothalamus or pituitary to affect luteinizing hormone (LH) levels. Alternatively, Leydig cells may be killed via an indirect action on Sertoli or germ cells since these three cell types depend on paracrine interactions for survival signals (Morris, 1996).

Some toxicants have been described that act directly on Leydig cells, inducing apoptosis and reducing serum testosterone levels (see section 4.4). Ethane dimethanesulphonate (EDS) has a unique cytotoxic action on the Leydig cells of the rat testis and consequently, has been used to investigate the physiological role of the Leydig cell. Early reports hinted that interstitial cells of the rat testis may undergo apoptosis in response to hypophysectomy or EDS (Morris *et al.,* 1986, Tapanainen *et al.,* 1993, Henriksen *et al.,* 1995) but the findings were far from conclusive. However, work from our laboratory (Morris *et al.,* 1997, Taylor *et al.,* 1998) has shown that Leydig cells will undergo apoptosis in response to EDS treatment both *in vitro* and *in vivo* and the effect is dose and time dependent. It remains to be determined if this apoptotic cell death of Leydig cells in response to EDS is purely toxicological or if it has some physiological significance.

4.3 APOPTOSIS IN THE NORMAL TESTES

The numbers of apoptotic cells in rodent testes are low but groups of spermatogonia linked by intercellular bridges undergo apoptosis synchronously (Allan *et al.,* 1992, reviewed in Sharpe, 1994). Indeed, the first clear description was provided by Huckins, 1978, although at the time the phenomenon of apoptosis had not been recognized. Primary and secondary spermatocytes as well as spermatids occasionally undergo apoptosis (Russel and Clermont, 1977, Kerr, 1992, Brinkworth *et al.,* 1995, Blanco-Rodriguez and Martinez-Garcia, 1996a). However, A1, Intermediate and type-B spermatogonia rarely degenerate (Huckins, 1978).

Removal of LH and follicle stimulating hormone (FSH) by hypophysectomy increases the number of degenerating pachytene spermatocytes (Russel and Clermont, 1977) via apoptosis (Tapanainen *et al.,* 1993, reviewed in Sharpe, 1994). This can be attenuated by addition of exogenous LH and FSH alone or in combination (Russel and Clermont, 1977, Tapanainen *et al.,* 1993). The observation that testosterone also prevents hypophysectomy-induced cell death (Tapanainen *et al.,* 1993) suggests a critical role for LH-stimulated testosterone secretion for the maintenance of germ cell viability.

The exact role of FSH in spermatogenesis is still uncertain (reviewed in Sharpe, 1994). FSH can prevent programmed cell death of rat pachytene spermatocytes and spermatids *in vitro* (Henriksen *et al.,* 1996) whilst at the same time stimulating proliferation of spermatogonia and preleptotene spermatocytes. *In vivo,* specific immunoneutralization of FSH induces apoptosis of spermatogonia and pachytene spermatocytes as early as 24 h after administration of the FSH antiserum (Shetty *et al.,* 1996).

In human testes, apoptosis occurs spontaneously as shown in tissues obtained after orchidectomy for prostate cancer (Brinkworth *et al.,* 1997, Woolveridge *et al.,* 1998a) or at autopsy following sudden traumatic death (Sinha Hikim *et al.,* 1998). Apoptosis occurs in a wide variety of germ cell types but not in Sertoli or Leydig cells and there is no significant relationship between the amount of apoptosis and either the age of men or testis weight (Brinkworth *et al.,* 1997). There are ethnic differences, with a higher incidence

of spermatocyte apoptosis in Chinese men compared to Caucasian men, which may explain their greater susceptibility to testosterone-induced spermatogenic suppression for contraceptive purposes (Sinha Hikim *et al.,* 1998).

Men with azoospermia or severe oligozoospermia have an increased frequency of apoptotic germ cells in their testicular biopsies in comparison to men with normal spermatogenesis, suggesting that programmed cell death may play a role in human male infertility (Lin *et al.,* 1997a, b). In addition, there is an increased frequency in the number of spermatozoa with apoptotic DNA in the ejaculates of infertile men in comparison to fertile controls (Gorczyca *et al.,* 1993, Baccetti *et al.,* 1996).

4.3.1 Genetic control of apoptosis in the normal testes

4.3.1.1 Bcl-2 family members

The role of Bcl-2 family members in the molecular regulation of apoptosis has been described in Chapter 1. In the rat testes, the two *bcl-2* transcripts present are highly expressed (Tilly *et al.,* 1995) but immature rat testis expresses the 7.5 kb species only. Bcl-2 protein is abundant in both the immature and adult rat testis (Woolveridge *et al.,* 1998b). Some groups have shown Bcl-2 immunoreactivity in human and mouse testes (Krajewski *et al.,* 1995, Woolveridge *et al.,* 1998a) whilst others have not (Knudson *et al.,* 1995, Furuchi *et al.,* 1996, Rodriguez *et al.,* 1997). In the former case, immunohistochemistry has localized Bcl-2 to the spermatids and mature sperm in the seminiferous tubules of the testis (Krajewski *et al.,* 1995). Bcl-2 knockout mice do not have any gonadal abnormalities although the grossly disturbed physiology, which includes growth retardation, lymphoid apoptosis and polycystic kidneys usually leading to death, makes it difficult to obtain meaningful data (Nakayama *et al.,* 1993, 1994, Kamada e*t al.,* 1995).

Bax is expressed as two transcripts in rat tissues, a predominant 1.0 kb form and a less abundant 1.5 kb species. The levels of these transcripts (Tilly *et al.,* 1995) and gene products (Woolveridge *et al.,* 1998b) are highest in reproductive tissues. Bax is present in the mouse and human testis (Knudson *et al.,* 1995, Woolveridge *et al.,* 1998a) although some studies show it is hardly detectable in the mouse (Rodriguez *et al.,* 1997). Immunostaining for Bax is seen only in the germinal cells located near the basement membrane (Krajewski *et al.,* 1994b). Bax-knockout males are infertile as a result of accumulation of atypical premeiotic germ cells, but no mature haploid sperm (Knudson *et al.,* 1995). *In situ* end labelling of apoptotic cells identified a large increase in the number of apoptotic cells in the Bax-deficient mice in comparison with control testes.

Two forms of *Bcl-x* transcript are expressed in the rat testis, a 2.7 kb species and a less abundant 3.5 kb form. A doublet of proteins with an apparent molecular weight of 29–31 kDa is present in the testis (Krajewski *et al.,* 1994a) which represents Bcl-XL and an additional 20 kDa band corresponding to Bcl-xs, is also present. Bcl-x is present in the

spermatocytes and spermatids of the human and mouse testis (Krajewski *et al.,* 1994a, Knudson *et al.,* 1995, Woolveridge *et al.,* 1998a) although, again, some studies have shown that it is hardly detectable in the mouse (Rodriguez *et al.,* 1997).

Bcl-w, a gene which promotes cell survival (Gibson *et al.,* 1996) is expressed in the elongating spermatids and Sertoli cells of the mouse testis (Ross *et al.,* 1998). Bcl-w knockout mice are sterile which is a result of progressive testicular degeneration (Ross *et al.,* 1998). The lack of Bcl-w results in a phenotype in which spermatocytes and spermatids are lost by apoptosis resulting in a Sertoli-cell only spermatogenic epithelium. Eventually there is almost complete loss of Sertoli cells and Leydig cell number is reduced although it is not clear if this is an indirect response to the lack of germ cells or a direct effect of the absence of Bcl-w.

Both the immature and adult rat testes express Bak and Bad (Woolveridge *et al.,* 1999). Bad is present in mice testes although Bak is not (Rodriguez *et al.,* 1997). In the human testis, Bak is associated with the Sertoli and Leydig cells (Krajewski *et al.,* 1996) whilst Mcl-1 is found only in the Leydig cells (Krajewski *et al.,* 1995). A new pro-apoptotic member of the Bcl-2 family, Bcl-2-related ovarian killer (Bok) is highly expressed in most reproductive tissues including the testis and could play an important role in spermatogenesis (Hsu *et al.,* 1997).

4.3.1.2 p53

The role of p53 in apoptosis has been described in detail in Chapter 1. In the testes, expression is mainly confined to the pachytene spermatocytes in the seminiferous tubules of normal rodents (Almon *et al.,* 1993, Rotter *et al.,* 1993, Schwartz *et al.,* 1994, Sjoblom and Lahdetie, 1996) with some expression in the spermatogonia and spermatids (Stephan *et al.,* 1996). p53 may play a role during meiosis in DNA repair and recombination events because the spermatogenic epithelium in p53 null mice contains multinucleated giant cells which are thought to be the result of the inability of the tetraploid pachytene spermatocytes to complete their meiotic division (Rotter *et al.,* 1993). However, the apoptotic elimination of spermatocytes with synaptic errors has recently been shown to be p53-independent (Odorisio *et al.,* 1998).

4.3.1.3 Fas

The testes express Fas ligand (Fas-L) at high levels (Griffith *et al.,* 1995, Suda *et al.,* 1995). Most reports have localized Fas-L to the Sertoli cells (Bellgrau *et al.,* 1995, French *et al.,* 1996, Lee *et al.,* 1997) although some have reported expression in the germ cells (Sugihara *et al.,* 1997). In contrast, some studies have shown that Fas-L expression is virtually absent in both the Sertoli cells and germ cells (Li *et al.,* 1997). We have shown that although FasL is expressed in the Sertoli cells, it is predominantly localized in the pachytene spermatocytes and spermatids (Woolveridge *et al.,* 1999). It is thought that the immune-privileged sanctuary within the testes is conferred by Fas-L expression (Bellgrau *et al.,*

1995). It is generally accepted with notable exceptions (Watanabe-Fukunaga *et al.,* 1992, Leithauser *et al.,* 1993) that Fas receptor (Fas-R) is expressed in the seminiferous tubules of the testis (Lee *et al.,* 1997, Li *et al.,* 1997, Sugihara *et al.,* 1997, Ogi *et al.,* 1998), in particular in spermatocytes (Lee *et al.,* 1997) and spermatids (Li *et al.,* 1997) with some reports also showing localization in the Sertoli cells (Sugihara *et al.,* 1997).

4.4 TOXICANT-INDUCED TESTICULAR APOPTOSIS

Many diverse toxicants cause apoptosis in the testes. Some may induce germ cell apoptosis directly whereas others act on the Leydig or Sertoli cells to cause androgen ablation and remove trophic support. Examples of such toxicants and a discussion of their site and mode of action is presented below and summarized in Table 4.1.

4.4.1 EDS

EDS is a unique testicular toxin with cytotoxic action confined almost exclusively to the Leydig cells (Morris *et al.,* 1986, Morris, 1996) (Table 4.1). EDS selectively eliminates both basal and LH-stimulated testosterone production. As with androgen withdrawal by other pharmacological/physiological manipulation, the predominant germ cell types undergoing apoptosis as a result of androgen withdrawal induced by EDS include pachytene spermatocytes and spermatids in stage VII of spermatogenic cycle (Henriksen *et al.,* 1995). This effect is suppressed by co-administration of testosterone.

During apoptosis of germ cells following androgen withdrawal there are no statistically significant changes in the levels of Bcl-XL, Bak or Bad in the rat testis although Bcl-XL levels do appear to decline (Woolveridge *et al.,* 1999). The decline in Bcl-XL levels might be explained on the basis that it is a survival gene product which might be expected to fall during apoptosis. However, the decline could be a reflection of the loss of Bcl-XL expressing spermatocytes and spermatids (Krajewski *et al.,* 1994a). The expression of Bax and Bcl-2 in the rat testes is up-regulated following withdrawal of androgen (Woolveridge *et al.,* 1999). A rise in Bcl-2 protein levels may reflect an increase in Bcl-2 production by the remaining mature germ cells perhaps as part of a survival mechanism to ensure that the atrophied tissue can respond to restimulation by androgen. At the same time, the pro-apoptotic gene Bax is increased to induce apoptosis. Selective enrichment of Bax-expressing cell types is an alternative explanation.

Despite the marked fall in androgen support to the spermatogenic epithelium following short-term anti-androgen treatment of human subjects, there is no significant change in the expression of the Bcl-2 family members. Similar to the rat, in the long-term androgen depleted human testes, Bcl-XL protein levels decline and Bcl-2 levels are elevated (Woolveridge *et al.,* 1998a). Withdrawal of androgen support to both the rat (Woolveridge *et al.,* 1999) and human (Woolveridge *et al.,* 1998a) testes has no effect on the expression of p53.

The expression of Fas-L declines dramatically 2 days following androgen withdrawal induced by EDS treatment and is barely detectable by 8 days (Woolveridge *et al.,* 1999). The initial drop in Fas-L expression is probably due to loss of Leydig cells within the testis because it is known that these cells also abundantly express Fas-L (Li *et al.,* 1997, Sugihara *et al.,* 1997, Taylor *et al.,* 1999). However, the significant further fall in Fas-L expression between 2 days and 8 days of EDS treatment must be due to changes in the seminiferous epithelium because Leydig cells are absent until at least 14 days after EDS treatment (Jackson *et al.,* 1986). This is confirmed by immunohistochemical data which shows that the number of germ cells expressing Fas-L and the intensity of Fas-L staining in the remaining germ cells is reduced at the later stages of androgen withdrawal (Woolveridge *et al.,* 1999). The expression of Fas-R also declines at 8 days following EDS administration. As with Bcl-XL, the decline in Fas-L and Fas-R expression following androgen ablation might be the result of the loss of the cell types expressing these genes. However, as the co-localization of Fas-L and Fas-R correlates with the germ cell types that die following androgen withdrawal, the potential does exist for apoptosis in the rat spermatogenic epithelium to be regulated by the Fas pathway. Surprisingly, the upregulation in these gene products at a time before the majority of apoptosis occurs, which has been observed in response to other testicular toxicants (Lee *et al.,* 1997), was not detected following androgen ablation.

Clusterin levels are not directly influenced by androgen levels (Frasoldati *et al.,* 1995, Woolveridge *et al.,* 1999). Clusterin mRNA levels are transiently elevated in the testis 6 h after EDS administration (Frasoldati *et al.,* 1999) perhaps as a secondary event to a direct effect of the alkylating agent on Sertoli cells. However, clusterin expression returns to basal levels and is unchanged until 21 days after EDS administration.

After hypophysectomy, the levels of the caspase substrate, poly (ADP ribose) polymerase (PARP) in the rat testis do not change despite loss of spermatocytes (Alcivar-Warren *et al.,* 1996). Also, short-term anti-androgen treatment in humans does not effect PARP protein levels in the testis (Woolveridge *et al.,* 1998a). In the chronically androgen depleted human testes PARP protein levels decline. This could be explained by the role of PARP in DNA repair so that levels are expected to be suppressed during apoptosis. However, as the cell types expressing PARP are lost in this essentially germ cell depleted testis, the levels in each individual cell may have remained unchanged.

Although the initial events by which EDS triggers the genetic programme of Leydig cell death are unknown at present, it could be suggested that the covalent binding of EDS to glutathione and its cellular depletion is important as this triggers apoptosis in response to cytotoxic treatments in other cells (Beaver and Waring, 1995, Sugimoto *et al.,* 1996). However, *in vitro* studies with Leydig cells suggest that the depletion of cellular glutathione actually protects the cells from EDS-induced death (Kelce and Zirkin, 1993, Kelce, 1994), which is consistent with the findings of one study showing that glutathione levels might not be important for Fas-mediated cell death (Marchetti

et al., 1997). Possibly EDS acts through inhibition of protein synthesis (Morris, 1996) as a result of the formation of an active complex with glutathione. Evidence for this comes from observations that cycloheximide, another protein synthesis inhibitor, can also induce an extremely rapid elevation of the protein components of the Fas system (Kimura and Yamamoto, 1997).

4.4.2 Estradiol

Estradiol is a natural hormone that, in excess, can act as a toxicant by causing endocrine disruption. In the testes, estradiol mimics some of the effects of hypophysectomy through the suppression of gonadotrophin production. As expected, the predominant cell types which undergo apoptosis in response to estradiol treatment include elongating and round spermatids and to a lesser extent, pachytene spermatocytes (Blanco-Rodriguez and Martinez-Garcia, 1996b, 1997). Cell death is suppressed to varying degrees by testosterone or a combination of FSH and LH (Blanco-Rodriguez and Martinez-Garcia, 1997). This apoptosis occurs before the germ cells detach themselves from the seminiferous epithelium which would suggest that germ cell detachment is not necessary for apoptosis to occur (Blanco-Rodriguez and Martinez-Garcia, 1998). Diethylstilbesterol (DES), an estrogen receptor agonist, induces regression of the testis in male Syrian hamsters. This is associated with an increase in apoptosis of spermatocytes and to a much lesser extent, spermatogonia (Nonclercq *et al.,* 1996).

4.4.3 PCBs

Polychlorinated biphenyls (PCBs) are a group of industrial chemicals with a myriad of uses. They are environmentally stable and bioaccumulate in marine, avian and mammalian species particularly in steroid producing organs such as the testes (Brandt, 1977) where they have detrimental effects upon male fertility (Bush *et al.,* 1986) (Table 4.1). It has been proposed that one of the anti-fertility mechanisms is to decrease testicular interstitial cell viability (Johansson, 1989, Kovacevic *et al.,* 1995). PCB169 acts at the aryl hydrocarbon receptor (Poland and Glover, 1977) and can decrease serum testosterone levels by more than 98% (Yeowell *et al.,* 1989). Preliminary data from our laboratory would suggest that *in vitro,* PCB169 can reduce hCG-stimulated testosterone production in Leydig cells at high concentrations. In addition, PCB169 will induce apoptosis of Leydig cells at higher doses. As the dynamics of cell death are different to EDS, the data would suggest that the mechanisms by which PCBs induce apoptosis are different from the alkylating action of EDS and may be related to their activity as aryl hydrocarbon receptor agonists (M.Taylor *et al.,* unpublished observations).

4.4.4 Methoxy acetic acid

One member of a family of ethylene glycol ethers whose use as organic solvents is widespread is 2-methoxy ethanol (2-ME) (Table 4.1). This includes incorporation into both domestic and industrial products such as textile dyes, printing inks and paints. There is concern for workers exposed to such chemicals, the majority of whom are male, because these compounds have the potential to produce toxicity in animals in organs that have active cell proliferation such as the bone marrow, thymus and testis (Balasubramanian *et al.,* 1994, Simialowicz *et al.,* 1994, Ku *et al.,* 1995). The active metabolite of 2-ME, 2-methoxy acetic acid (2-MAA), induces lesions in the testes of rats and guinea pigs which are a result of death of pachytene spermatocytes (Anderson *et al.,* 1987) by apoptosis (Brinkworth *et al.,* 1995, Ku *et al.,* 1995), although pachytene spermatocyte degeneration in rats and guinea pigs differs in onset, severity and morphological characteristics (Ku *et al.,* 1995). Apoptosis of rat spermatocytes is very extensive following 2-MAA treatment *in vivo* and dying cells often form rings around the edges of the seminiferous tubule (Clark *et al.,* 1997). The expression of clusterin in the rat testis is elevated in response to 2-MAA induced apoptosis and accumulates in the cytoplasm of dying pachytene spermatocytes as early as 6 h post-2-MAA administration (Clark *et al.,* 1997).

A divalent metal cation-dependent endonuclease activity is present in 2-MAA treated rat testes and increased intracellular calcium might be a stimulus for 2-MAA-induced spermatocyte apoptosis (Ku *et al.,* 1995). An 18 kDa endonuclease was isolated from the pachytene spermatocytes of 2-MAA treated rats (Wine *et al.,* 1997) which is able to cleave DNA into multiples of 200 base pairs. Furthermore, dihydropyridines such as nifedipine or verapamil are able to attenuate 2-MAA-induced apoptosis of pachytene spermatocytes *in vitro* (Li *et al.,* 1996, 1997). All of these compounds inhibit calcium movement through plasma membranes. Drugs which inhibit calcium movement from intracellular stores or block the effect of inositol tri-phosphate have no effect on 2-MAA-induced apoptosis (Li *et al.,* 1997). However, immunohistochemical staining for the dihydropyridine receptor in rat testicular sections proved to be negative and there is no evidence for an increase in intracellular free calcium concentration in germ cells following 2-MAA treatment (Li *et al.,* 1997). The protective effect of calcium channel blockers against the 2-MAA-induced spermatocyte apoptosis is therefore probably not due to blockade of DHP-sensitive calcium channels.

4.4.5 2,5-Hexanedione

A metabolite of the commonly used solvents n-hexane and 2-hexanone, 2,5-hexanedione (2,5-HD) is a Sertoli cell toxicant (Table 4.1) which indirectly causes irreversible and widespread germ cell loss and testicular atrophy in rats. The irreversible nature of 2,5-HD toxicity is probably due to direct action that reduced the number of stem cells; those

that are remaining appear apoptotic (Allard and Boekelheide, 1996). After administration of 1% 2,5-HD in drinking water over 5 weeks, there is a marked increase in germ cell apoptosis (Blanchard *et al.,* 1996). There is also a differential sensitivity of germ cells to toxicant exposure with apoptotic spermatids appearing first followed by apoptotic spermatogonia and spermatocytes.

4.4.6 1,3-Dinitrobenzene/nitrobenzene

The volatile nitroaromatics have a variety of industrial uses and are therefore an occupational hazard to humans. Another Sertoli cell toxicant (Table 4.1), 1,3-dinitrobenzene (DNB), induces apoptosis in the rat testis with a very fast onset following administration *in vivo* (Strandgaard and Miller, 1998) reaching a maximum at 24 to 48 h. The predominant cell types affected are the pachytene spermatocytes. Nitrobenzene affects the same cell types over a similar time frame (Shinoda *et al.,* 1998).

4.4.7 Mono-(2-ethylhexyl) phthalate

Phthalate esters are plasticizers present in food packaging and therefore are ideally placed for ingestion by humans. Mono-(Z-ethylhexyl) phthalate (MEHP) is a Sertoli cell toxicant (Table 4.1) which also induces testicular germ cell apoptosis *in vivo* in young rats (Richburg and Boekelheide, 1996). Three hours following MEHP administration, Sertoli cell vimentin filaments collapse although tubulin and actin are unaffected. The major cell types undergoing apoptosis following MEHP treatment are the spermatocytes (Lee *et al.,* 1997). Fas is implicated in mediating the effects of MEHP (Lee *et al.,* 1997). Maximal induction of Fas-L and Fas-R expression is after 6 h or 12 h, respectively and the localization pattern of Sertoli cell Fas-L was changed 12 h after MEHP-exposure to a diffuse cytoplasmic staining pattern, particularly at sites of Sertoli cell-germ attachment (Lee *et al.,* 1997). These observations suggest that under normal physiological conditions the Sertoli cell mediates the death of Fas-R bearing germ cells by expressing Fas-L on its cell membrane (Figure 4.2). The initial disruption of the Sertoli cell-germ cell physical contact may result in the uncoupling of the Fas-mediated signal transduction process between these cells leading to a decrease in germ cell apoptosis but at later stages the expression of Fas-L increases to eliminate damaged germ cells. This may be mediated by the generation of a soluble form of Fas-L from Sertoli cells (Figure 4.2C).

4.4.8 Cadmium

Exposure to cadmium in dusts and fumes is an occupational hazard for some people. In rodents, cadmium chloride can induce apoptosis in testes *in vivo* at a dose level of 0.03 mmol/Kg and over a time period of 48 hours (XU *et al.,* 1996, Yan *et al.,* 1997)

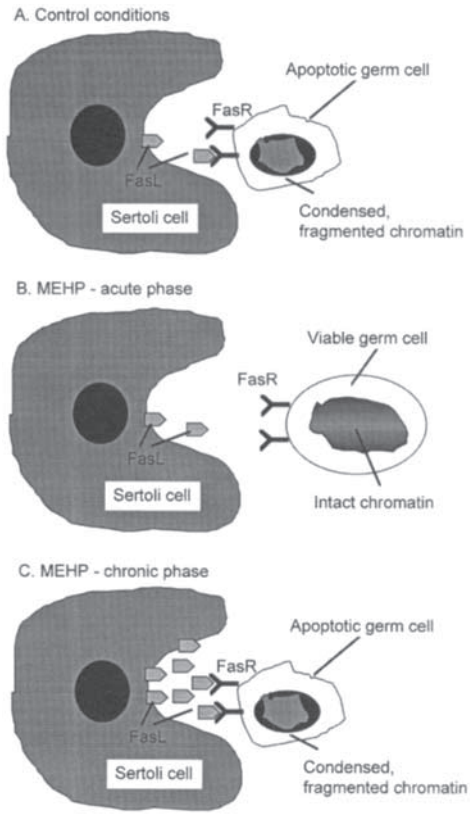

Figure 4.2 Schematic diagram of the interaction between a Sertoli cell and a germ cell showing induction of apoptosis by MEHP involving release of Fas-L.

(Table 4.1). Cadmium chelating agents can suppress most, if not all, of this apoptosis depending on the time between administration of the cadmium and administration of the chelating agent. Selenium can offset cadmium-induced toxicity in many tissues including the testes although the exact mechanism is unclear but like chelating agents, selenium suppresses cadmium-induced testicular apoptosis if administered prior to or after cadmium (Jones *et al.,* 1997).

4.4.9 Phosphamidon

Phosphamidon is one member of the organophosphate family of insecticides which is used widely in agriculture. There is almost a complete absence of differentiating spermatids and spermatozoa in the testes of male rats fed with drinking water containing

TABLE 4.1
Toxicants and hormones that induce testicular apoptosis

Toxicant	Site of action	Mode of action	Reference
Estradiol	• estrogen receptor (ER)	• androgen ablation; gonadotrophin ↓ • prevented by testosterone or FSH/LH	Blanco-Rodriguez and Martinez-Garcia, 1997, 1998 Nonclercq *et al.*, 1996
Diethyl stibestrol (DES)	• estrogen receptor (ER)	• androgen ablation; gonadotrophin ↓	Nonclercq *et al.*, 1996
Ethane dimethanesulfonate (EDS)	• Leydig cell toxicant	• Testosterone ↓ • loss of trophic support ⇒ germ cell apoptosis	Morris *et al.*, 1986, 1997 Morris, 1996 Taylor *et al.*, 1998
2-Methoxy ethanol (2-ME)	• Ca^{2+} regulation?	• extensive apoptosis of spermatocytes • clusterin expression ↑ • Ca^{2+}↑; ⇒ activation of endonuclease	Ku *et al.*, 1995, Clark *et al.*, 1997, Li *et al.*, 1996, 1997, Wine *et al.*, 1997
2,5-Hexanedione (2,5-HD)	• Sertoli cell toxicant	• causes widespread germ cell loss	Allard and Boekelheide, 1996, Blanchard *et al.*, 1996
1,3-Dinitrobenzene (DNB)	• Sertoli cell toxicant	• causes widespread germ cell loss	Strandgaard and Miller, 1998, Shinoda *et al.*, 1998
Mono-(2-ethylhexyl) phthalate (MEHP)	• Sertoli cell toxicant	• causes widespread germ cell loss • FasL implicated in toxicity	Richburg and Boekelheide, 1996, Lee *et al.*, 1997 (see Figure 4.2)
Cadmium		• causes germ cell apoptosis • Offset by cadmium chelating agents or selenium	Xu *et al.*, 1996, Yan *et al.*, 1997, Jones *et al.*, 1977
Phosphamidon		• ablation of differentiating spermatids	Akbarsha and Sivasamy, 1997
Polychlorinated biphenyls (PCBs)	• aryl hydrocarbon receptor	• testosterone ↓ • reduced viability of interstitial cells • loss of trophic support?	Brandt, 1977, Bush *et al.*, 1986, Johansson, 1989, Kovacevic *et al.*, 1995, Poland and Glover, 1977, Yeowell *et al.*, 1989
Gossypol	• inhibits protein kinase C activity	• proliferation ↓ • cell death ↑ • potential male contraceptive	Lui *et al.*, 1985, Teng, 1995

phosphamidon at 35 ppm for 30 days (Akbarsha and Sivasamy, 1997) and numerous uni-and multi-nucleate giant cells with typical apoptotic morphology.

4.5 TESTICULAR APOPTOSIS AND DRUGS

Apoptotic death in the testes is caused by two classes of prescription drugs, the male contraceptive and the DNA-damaging chemotherapeutic agents (Table 4.1).

4.5.1 Gossypol

Gossypol acetic acid is a drug that has undergone trials as a possible male contraceptive (Lui *et al.,* 1985). It inhibits spermatogenesis in mammalian species by preventing the proliferation of germ cells. Gossypol causes malformations in spermatozoa which include alterations in the chemical composition of chromatin, DNA strand breakage, changes in the shape of the sperm head and a reduction in sperm motility which all result in the death of sperm cells. Gossypol inhibits protein kinase C activity and induces apoptosis in cultured rat spermatocytes which is prevented by activators of protein kinase C (Teng, 1995), which would suggest that maintenance of protein kinase C activity is needed to protect spermatocytes from apoptosis.

4.5.2 Chemotherapeutic drugs

The majority of chemotherapeutic agents kill cells in proliferating tissues by damaging DNA. Thus, the testes are very vulnerable to toxicity from this class of agents. Vincristine and vinblastine are microtubule disrupting agents that play an essential role in combination cancer chemotherapy. However, they disrupt spermatogenesis resulting in the formation of multinucleated giant cells. Vincristine affects spermatogenesis in a dose and time-dependent manner at mitotic and meiotic stages resulting in the cells arrested in division dying by apoptosis (Akbarsha *et al.,* 1996).

Mitomycin C (Kobayashi, 1960) alkylates DNA and affects DNA synthesis (Iyer and Szybalski, 1963) to cause chromosome aberrations and dominant-lethal mutations in mouse spermatogonia and spermatocytes. At higher doses, these cells die *in situ* by apoptosis (Nakagawa *et al.,* 1997). Apoptotic elimination of testicular germ cells as a result of anti-cancer therapies may serve to protect subsequent generations from the genotoxic effects of these drugs. Cyclophosphamide, is another cytotoxic DNA and protein alkylating agent that is used in the treatment of cancer but also causes infertility. Cyclophosphamide induces apoptosis in germ cell cells in the seminiferous epithelium of the rat testis in a time-dependent manner reaching a peak at 12 h after a 70 mg/kg dose and declining thereafter, reaching control levels at 48 h (Cai *et al.,* 1997). Lower doses of Cyclophosphamide do not induce significant levels of apoptosis but produce

transgenerational mutations. The cell types affected include spermatogonia and spermatocytes in all stages of the spermatogenic cycle.

The topoisomerase II inhibitors, etoposide and adriamycin, cause DNA damage by inducing strand breaks. They are well known anti-cancer agents that cause the elimination by apoptosis of spermatogonia, zygotene and early pachytene spermatocytes and meiotically dividing spermatocytes (Sjoblom *et al.,* 1998). Etoposide and adriamycin have no effect on p53 or p21 (WAF1) levels (Sjoblom *et al.,* 1998).

4.6 SUMMARY

Clearly a large number of industrial and therapeutic compounds as well as environmental toxicants can disrupt spermatogenesis and induce programmed cell death in the germ and somatic cells of the testis. However, the mechanisms of the induction of germ cell apoptosis are unclear. Further understanding of the toxicology of the testis, future treatments for male infertility and the development of male contraceptives may rely upon a more detailed understanding of the genetic control of toxicant-induced programmed cell death in the testis.

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CHAPTER 5

Apoptosis in Female Reproductive Toxicology

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Contents

5.1 Introduction
5.2 Ovarian physiology
5.3 Apoptosis and ovarian toxicology
5.4 Summary

5.1 INTRODUCTION

Although the hormonal interplay between components of the human female reproductive system is extremely complex, each organ or tissue that comprises the reproductive apparatus in women contributes towards a single goal: the birth of healthy offspring. A precise and synchronized monthly series of events both produce a mature egg for fertilization and embryogenesis and prepare the uterine endometrial lining for implantation should pregnancy occur. However, this process is unfortunately very sensitive to disruption by a host of environmental insults. Furthermore, due to the active involvement of multiple tissues in achieving successful conception and pregnancy, reproductive toxicology in females covers a vast expanse of research and clinical arenas. Because of this, and the fact that generation of a competent germ cell, an ovulated egg in females, is the first step in species propagation, the present discourse will focus solely on the impact of environmental and clinical toxicants on the ovary. For reviews of toxicant effects on other target tissues associated with female reproductive function, the reader is referred elsewhere (for examples, see Sastry, 1991, Mattison, 1993, Kamrin *et al.,* 1994, Sharara *et al.,* 1998, Spielmann, 1998).

The ovaries of all mammalian species are the powerhouses behind reproductive performance, providing not only the gametes needed for fertilization but also a long list of hormones and bioactive peptides that co-ordinate intraovarian events and those taking place in the hypothalamic-pituitary axis, oviducts and uterus. Together, these are needed for oocyte maturation, ovulation and fertilization, as well as for embryo implantation and gestation. At its most basic level, the female gonad can be roughly divided into four major functional cell types: germ cells (oogonia and oocytes), granulosa cells and theca-interstitial cells of the follicle and luteal cells of the corpus luteum formed following ovulation (Figure 5.1). The ovary is a prime target for disrupted function following toxicant exposure from its

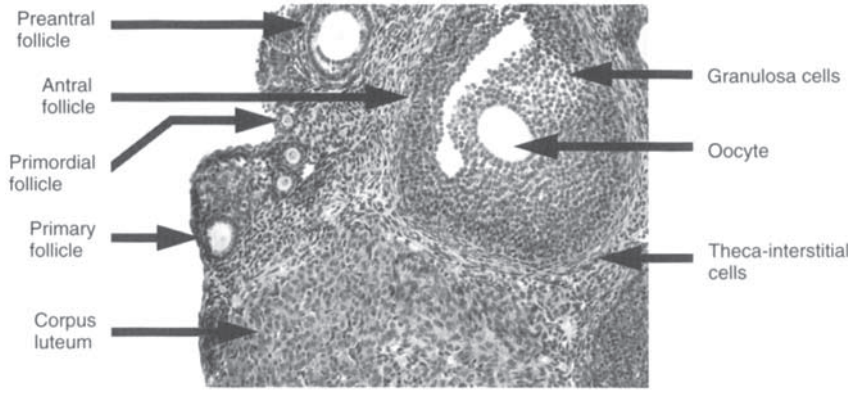

Figure 5.1 Diagram of the ovary. Follicles in various stages of development are scattered throughout.

earliest stages as a presumptive gonad (genital ridge) housing primordial germ cells to its later stages during the menopausal transition. Furthermore, the impact of any given toxicant on ovarian function and infertility reflects the stage(s) of follicles that are vulnerable to the specific insult. Therefore, this chapter will begin with a brief overview of ovarian development, with particular emphasis on germ cell and follicle dynamics as well as on the role of apoptosis as a normal mechanism by which oocytes, follicles and corpora lutea are deleted. This section will be followed by a more intensive discussion of the literature implicating premature or inappropriate apoptosis as a common denominator in many paradigms of ovarian toxicity.

5.2 OVARIAN PHYSIOLOGY

5.2.1 Apoptosis and foetal oogenesis

During early embryogenesis in mammals, a relatively small number of primordial germ cells migrate from the yolk sac through the mesentery to the presumptive gonads or genital ridges where a programme of rapid germ cell mitosis is set in motion (Byskov, 1986, Buehr, 1997) (Figure 5.2). In the human female foetus, peak numbers of germ cells

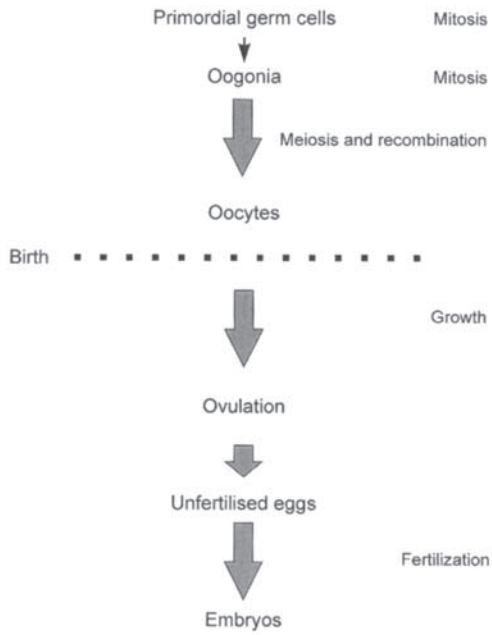

Figure 5.2 Landmarks of oogenesis. Progression from primordial germ cells during foetal development and from oocytes to fertilized eggs in sexually mature adults.

(approximately 7×10^6), referred to as oogonia, are produced by the twentieth week of gestation through clonal expansion (Baker, 1963). Many of these oogonia begin the first steps of meiosis, passing through the leptotene, zygotene and pachytene phases, but become arrested in the last step of meiotic prophase (the diplotene stage) during folliculogenesis, at which time the arrested germ cell (now referred to as an oocyte) is surrounded by a single layer of specialized somatic cells termed granulosa cells to form a primordial follicle (Byskov, 1986). In almost all mammalian species, female germ cell mitosis ceases by the time of birth, providing for a finite reserve of oocytes that cannot be replenished in postnatal life. It is this distinguishing feature of a nonrenewable germ cell population that places women, as opposed to men who maintain active spermatogenesis throughout their adult lives, at such a tremendous risk for irreversible reproductive damage upon toxicant exposure.

Female germ cell dynamics is further complicated by the high rate of prenatal oogonium and oocyte attrition known to occur naturally in essentially all mammalian species. For example, in the human female foetus only $1–2\times10^6$ of the 7×10^6 germ cells present at the twentieth week of gestation remain in the ovaries at birth (Baker, 1963, Forabosco *et al.,* 1991), with the other two-thirds of the germ cells being lost via apoptosis (De Pol *et al.,* 1997). Similar events occur in the foetal ovaries of rodents (Borum, 1961, Beaumont and Mandl, 1962, Coucouvanis *et al.,* 1993, Ratts *et al.,* 1995), thus offering relevant and easily-studied model systems to explore the basis of prenatal germ cell death. In fact, the recent development of an organ culture system for maintaining foetal mouse ovaries *in vitro* has already provided intriguing new clues as to the regulation of oogonium and oocyte apoptosis (Morita *et al.,* 1999). This latter study identified the existence of a complex interplay between growth factors and cytokines in determining germ cell fate, and further showed that activation of a key signal transduction enzyme, phosphatidylinositol 3'-kinase (PI3K) (see Chapters 1 and 3), is a critical step for diverse endocrine factors to promote foetal ovarian germ cell survival. These findings of Morita *et al.* (1999) are in agreement with a number of reports from analyses of various somatic cell lineages that have established a central role for PI3K in transducing survival signals to central regulatory steps in the apoptotic cell death pathway governed by Bcl-2 family members (Datta *et al.,* 1997, Kennedy *et al.,* 1997) (Chapter 1).

Aside from this recent investigation (Morita *et al.,* 1999), very little is currently known of the potential involvement of apoptosis-regulatory molecules as determinants of oogonium and oocyte fate during gametogenesis and folliculogenesis. A possible connection between Bcl-2 and ovarian germ cell survival was established several years ago in studies showing that young adult female mice lacking functional expression of the *bcl-2* gene (gene knockout) possess a significantly reduced number of oocyte-containing primordial follicles (Ratts *et al.,* 1995). Although published evidence of *bcl-2* gene expression in the developing foetal ovaries of any species is currently lacking, the conclusions drawn from the analysis of Bcl-2-deficient mice are supported by findings of polyadenylated

bcl-2 mRNA transcripts in postnatal murine oocytes (Jurisicova *et al.,* 1998). Another set of data derived from gene knockout mice, in this case animals lacking expression of functional Bax protein (Knudson *et al.,* 1995), has revealed that Bax may be dispensable for proper execution of apoptosis in the foetal ovarian germ line since the oocyte reserve endowed at birth (which reflects the magnitude of prenatal germ cell attrition) is identical in wild-type and Bax-null females (Perez *et al.,* 1999). However, during postnatal life Bax-deficient female mice exhibit many other pronounced defects in ovarian cell apoptosis (Perez *et al.,* 1999), including germ line resistance to apoptosis induced by toxicant exposure (Perez *et al.,* 1997), that will be expanded on in sections 5.2.2 and 5.3.1 below.

Lastly, a recent set of observations derived from a third gene knockout mouse line has provided the first evidence of a role for caspases (see Chapter 2) as final executioners of germ cell apoptosis during foetal ovarian development. This study reported that female mice lacking expression of functional caspase-2 (previously referred to as Ich-1; Wang *et al.,* 1994) are born with a significantly larger endowment of oocyte-containing primordial follicles when compared with wild-type sisters (Bergeron *et al.,* 1998). These data are consistent with preliminary observations that peptide inhibitors of caspases effectively suppress apoptosis in germ cells of cultured foetal mouse ovaries (Morita *et al.,* 1999a), although the exact caspase family member(s) inhibited by these peptides remains to be clarified. Similar to the comments made regarding Bcl-2, essentially nothing is known of the expression patterns of caspases in the developing foetal ovaries. Nonetheless, it is important to note that polyadenylated *caspase-2* mRNA transcripts are present in postnatal murine oocytes (Jurisicova *et al.,* 1998), consistent with the idea that caspase-2-null oocytes possess a survival advantage *in vivo* (Bergeron *et al.,* 1998). These findings therefore set the stage for the hypothesis that caspases, like Bcl-2 and related proteins, are indeed integral components of a genetic programme of cell death responsible for executing apoptosis in the female germ line (Tilly *et al.,* 1997, Morita and Tilly, 1999).

5.2.2 Postnatal ovarian development, follicle atresia and the menopause

For those oocytes that escape death during foetal ovarian development and thus become endowed as the primordial follicle stockpile, there are two possible fates. The first, which happens to a small percentage of the total germ cell pool, is development of the oocyte and the follicle through successive maturational stages (primordial, primary, preantral, antral and, finally, preovulatory). This is followed by release of the oocyte from the preovulatory follicle(s) at ovulation into the reproductive tract for possible fertilization. It is important to point out that along the pathway of maturation, the susceptibility of the oocyte and the supporting somatic cells (granulosa cells and, in latter stages of follicle development, theca-interstitial cells) to damage by a number of toxicants appears to decrease, possibly reflecting the enhanced ability of the increasing number of somatic

cells within developing follicles to neutralize potential threats to the oocyte housed within. The second possible fate of oocytes that comprise the primordial follicle reserve is apoptosis, an event that can either trigger or occur as a consequence of a normal degenerative process referred to as follicular atresia.

For as yet unknown reasons, the vast majority of follicles endowed in the ovary at birth will undergo atresia at some point in their developmental programme, thus never producing an ovulated egg for fertilization (Gougeon, 1996, Tilly, 1996). Atresia can occur at any point in the maturational pathway of follicles, although the cellular site of initiation of the event differs quite markedly dependent upon the developmental stage of the follicle. In quiescent (primordial) and slowly-growing immature (primary, early preantral) follicles, fragmentation and death of the oocyte appears prior to any discernible sign of somatic cell demise, suggesting that apoptosis of the germ cell drives atresia. In contrast, follicles progressing through the latter stages of maturation (late preantral, antral) that acquire dependency upon pituitary gonadotropin hormones, such as follicle-stimulating hormone (FSH), for continued survival and development undergo atresia due to apoptosis in the granulosa cell compartment of the follicle (Hughes and Gorospe, 1991, Tilly *et al.,* 1991, Chun *et al.,* 1994, Tilly *et al.,* 1995a, Tilly, 1998). Without direct support from and communication with its surrounding granulosa cells, the oocyte of the dying follicle eventually degenerates, removing itself irreversibly from the ovulatory pathway.

The impact of atresia on the fertile lifespan of the female is enormous when one considers, for example, that of the $1–2\times10^6$ primordial follicles endowed in the human ovaries at birth, up to one-half of these may be atretic (Baker, 1963). Furthermore, the number of follicles in the human female drops to less than 4×10^5 by the time of puberty, arguing that atresia claims over one-half to three-quarters of the oocytes before reproductive life even begins in women. Atresia remains a prominent feature of cyclic ovarian function until nearly complete exhaustion of the follicle reserve is observed at the time of the menopause (Richardson *et al.,* 1987, Tilly and Ratts, 1996).

In this regard, mounting evidence from gene expression and cell culture analyses implicate a number of apoptosis-regulatory molecules as principal signalling factors (Tilly *et al.,* 1997, Tilly, 1998) (Table 5.1). Expression of the pro-apoptotic protein encoded by the *bax* gene has proven to be tightly correlated with, if not absolutely required for, normal ovarian cell death to proceed. For example, *bax* transcripts accumulate in rat granulosa cells of early antral follicles on the verge of atresia, whereas provision of a survival factor such as exogenous gonadotropin *in vivo* reduces *bax* expression concordant with a suppression of granulosa cell apoptosis and follicular atresia (Tilly *et al.,* 1995a). Similar observations of a strong correlation between expression of the Bax protein, as determined by immunohistochemical analysis, and follicular atresia in the human ovary (Kugu *et al.,* 1998), emphasize the evolutionarily-conserved nature of the ovarian cell death programme.

The role of Bax in mediating granulosa cell demise was unequivocally established by morphological assessments of ovaries from young adult Bax-deficient female mice

Molecule	Function	Reference
p53 (see Chapter 1)	Role in sensing DNA damage. Transcription factor.	Tilly *et al.*, 1995b, Hosokawa *et al.*, 1998
Ceramide (see Chapter 1)	Role in signalling execution.	Witty *et al.*, 1996
Reactive oxygen species	Role in signal transduction.	Tilly and Tilly, 1995
Bcl-2 family members (see Chapter 1)	Central interpreters of signals for cell survival. Bax is strongly implicated in ovarian apoptosis.	Tilly *et al.*, 1995a, Knudson *et al.*, 1995, Kugu *et al.*, 1998, Perez *et al.*, 1999
Apaf-1	Couples Bcl-2 family function to caspase activation.	Robles *et al.*, 1999
Caspases (see Chapter 2)	Executioners.	Flaws *et al.*, 1995, Maravei *et al.*, 1997, Boone and Tsang, 1998, Kugu *et al.*, 1998
Serine proteases	Executioners	Trbovich *et al.*, 1998

TABLE 5.1
Molecules implicated in the regulation of follicular atresia

(Knudson *et al.*, 1995) which revealed the presence of numerous maturing follicles that presumably failed to properly complete the atresia process. A more extensive evaluation of the reproductive features of Bax-null female mice documented a significantly reduced incidence of primordial and primary follicle atresia, leading to a 3-fold surfeit of oocyte-containing primordial follicles, in young adult females (Perez *et al.*, 1999). These findings of a larger ovarian follicle reserve predicted that ovarian senescence would be dramatically delayed, if not absent, as Bax-deficient females reached advanced chronological ages. This postulate was confirmed by evidence that ovaries of aged Bax-null females do in fact retain large numbers of follicles at all stages of development, including large estrogenic antral follicles capable of sustaining uterine hypertrophy (Perez *et al.*, 1999). Aside from the obvious implications of this work, the study by Perez *et al.* (1999) has substantiated the relevance of using the power of mouse genetics to elucidate basic mechanisms responsible for follicle loss leading to the menopause.

5.2.3 The corpus luteum and pregnancy maintenance

During the periovulatory period, initiated by the surge of luteinizing hormone from the pituitary gland, granulosa and theca-interstitial cells of the preovulatory follicle(s) undergo a rapid transformation to luteal cells. This event is marked by the shift in steroidogenesis from principally estrogen to progesterone, the latter steroid being absolutely necessary for the maintenance of at least the early gestation period following implantation of the

conceptus if fertilization of the ovulated egg occurs (Patton and Stouffer, 1991). The corpus luteum has a pre-programmed lifespan that, unless informed otherwise by as yet incompletely understood mechanisms, ends in collapse of the tissue several days after being formed. The process of corpus luteum regression, or luteolysis, is accomplished in two not necessarily independent phases, the first demarcated by a loss of steroidogenic capacity followed by a second phase of structural involution (Rueda *et al.,* 1997a). Of course, one would predict that impaired progesterone output does not have to precede structural collapse since any agent, toxicant or otherwise, capable of triggering apoptosis in luteal cells would cause an abrupt loss in steroid production by this ovarian structure. As mentioned earlier, such a scenario would place the gestating foetus in grave danger of being aborted since the uterine lining is critically dependent upon ovarian steroid support for maintenance during pregnancy.

Work from a number of laboratories has shown that apoptosis is a prominent feature of structural luteolysis in various mammalian species (Juengel *et al.,* 1993, Dharmarajan *et al.,* 1994, Shikone *et al.,* 1996), as well as postovulatory follicular tissue resorption in the avian ovary (Tilly *et al.,* 1991). The endocrine regulation of this process is probably species-specific, with human chorionic gonadotropin (Dharmarajan *et al.,* 1994), prolactin (Matsuyama *et al.,* 1996), estradiol (Goodman *et al.,* 1998), progesterone (Gaytan *et al.,* 1998) and prostaglandin-$F_{2\alpha}$ (Rueda *et al.,* 1999) identified among others as potential candidates for controlling luteal cell fate. As recently reviewed (Rueda *et al.,* 1997a), the intracellular events set in motion in luteal cells by changes in the extracellular environment are probably the same as those activated in other cell types during apoptosis. For example, an alteration in the intracellular reduction-oxidation state leading to oxidative stress probably accompanies cell death during luteolysis (Rueda *et al.,* 1995, Dharmarajan *et al.,* 1999). Furthermore, altered expression patterns of key apoptosis-regulatory molecules, such as Bcl-2 family members and caspases, have been linked to luteal regression (Rueda *et al.,* 1997b, 1999, Goodman *et al.,* 1998, Dharmarajan *et al.,* 1999).

5.3 APOPTOSIS AND OVARIAN TOXICOLOGY

Since the number of chemicals that adversely affect female reproductive performance at the level of ovarian dysfunction is far too great to address in any one chapter (for examples, see Bishop *et al.,* 1997a,b), four classes of toxicants linked to apoptosis induction in the ovary have been selected for discussion. As a preface, it should be stressed that the stage of follicular development affected by exposure to any given toxicant determines its impact on female reproductive function. For example, chemicals that destroy growing and preovulatory follicles cause temporary infertility since these damaged follicles can be replaced by recruitment from the large pool of primordial and primary follicles present in the ovaries. In contrast, toxicants that damage or destroy primordial or primary follicles lead to early menopause and permanent infertility as these follicles are set as a finite

number at birth and cannot be replaced (see section 5.2). Of great concern, destruction of primordial follicles may not be detected for weeks, months or even years after exposure to xenobiotics since oestrous or menstrual cyclicity may not be affected until the entire pool of growing follicles has been depleted from the ovary. Therefore, risk assessment for environmental threats to the primordial follicle stockpile is extremely difficult to pursue since the only reliable method to detect such damage is serial section analysis of the ovary for total follicle counts (Bolon *et al.,* 1997, Bucci *et al.,* 1997). As a final comment here, the potential role of apoptosis as a mediator of chemical-induced ovarian damage is a very new concept that has not received sufficient research attention. Therefore, some of what is to follow is hypothetical but supported by published information where possible.

5.3.1 Dioxin (2, 3, 7, 8-tetrachlorodibenzo-p-dioxin or TCDD)

Dioxin is one of the most notorious environmental toxicants known (Poland and Knutson, 1982, Silbergeld, 1995). In humans, TCDD has been found at significant levels in ovarian follicular fluid (Tsutsumi *et al.,* 1998) but the consequences of this ovarian TCDD exposure remain to be fully clarified. In rats *in vivo,* TCDD causes abnormal estrous cyclicity and impaired ovulation rates (Li *et al.,* 1995a, Lu *et al.,* 1995) and reduces the numbers of preantral and antral ovarian follicles (Heimler *et al.,* 1998a). Somewhat surprisingly, the reduction in follicle number noted by Heimler *et al.* (1998a) was not associated with an increased incidence of apoptotic cell death in those follicles, raising the possibility that the primordial and/or primary follicle pool (which is the source of maturing preantral and antral follicles) is actually the target of TCDD action. However, previous work has established that primordial oocyte destruction does not occur in female mice following *in vivo* TCDD exposure (Mattison and Nightingale, 1980), leaving open the question as to exactly how TCDD causes a reduction in ovarian follicle numbers.

Species-specific differences in the ovarian cellular response to TCDD could explain the potentially discordant data regarding follicle destruction in mice and rats, and a recent publication supports this possibility. Using cultured human granulosa-luteal cells obtained from follicular aspirates of patients undergoing assisted reproductive technologies, Heimler *et al.* (1998b) reported that dioxin treatment caused a time- and dose-dependent increase in the incidence of apoptosis. The mechanisms underlying the apoptotic response to TCDD were unfortunately not elucidated, although TCDD was noted to also significantly attenuate estrogen production by reducing androgen precursor availability in cultured human granulosa-luteal cells (Heimler *et al.,* 1998b). In light of recent investigations identifying estradiol as a luteal cell survival factor in the rabbit (Goodman *et al.,* 1998), it is possible that the ability of dioxin to suppress estrogen biosynthesis in human granulosa-luteal cells serves as at least one initiating event that leads to apoptosis. Furthermore, in cultured human granulosa-luteal cells TCDD is known to modulate the activity of a number of protein kinases involved in cell signalling (Enan *et al.,* 1996a) and to interfere with

glucose transport (Enan *et al.,* 1996b); offering other possible sites of normal cellular function that may be disrupted by TCDD leading to apoptosis. Whatever the case, it is clear that additional studies are needed to fully understand the consequences of dioxin exposure on ovarian function in general and on ovarian cell death in particular.

5.3.2 Polycyclic aromatic hydrocarbons (PAH)

Polycyclic aromatic hydrocarbons are formed as a result of the incomplete combustion of organic compounds, most notably fossil fuels such as wood and coal (Committee on Biological Effects of Atmospheric Pollutants, 1972). Benzo[a]pyrene (BAP), a prototypical PAH, is released into the air at an estimated level of 1.8 million pounds per year, primarily derived from coal refuse piles, abandoned coal mines, coke manufacture, and residential combustion of bituminous and anthracite coals. Occupational exposure to PAH is also of concern, particularly for workers at coal tar and aluminum production plants, road and roof tarring operations, and municipal incineration sites that burn carbonaceous materials (National Occupational Exposure Survey 1981–1983). It should be noted that a primary route of human exposure to PAH is also via cigarette and cigar smoke (Mattison and Thorgeirsson, 1978), as levels of BAP and dimethylbenz[a]anthracene (DMBA) have been estimated at 0.1 mg and 4 mg per cigarette, respectively (World Health Organization/International Agency for Research on Cancer, 1973). A link between maternal cigarette smoking during pregnancy and reduced fecundity of female offspring exposed *in utero* has been reported (Weinberg *et al.,* 1989). These findings, taken with data which indicate that women who smoke undergo the menopause at an earlier age (Mattison *et al.,* 1989), strongly suggest that chemicals present in cigarette smoke impair foetal oogenesis and destroy pre-existing germ cells established in the ovary. These epidemiological findings that implicate PAH as ovotoxicants in humans are fully supported by experimental data obtained through analysis of the actions of BAP and DMBA in numerous animal studies. Massive foetal germ cell loss has been reported to occur in pregnant female mice injected with BAP (Mackenzie and Angevine, 1981). These findings have been reinforced by several studies documenting the effects of BAP and related PAHs (such as DMBA and 3-methylcholanthrene) on oocyte loss and disrupted follicular dynamics in postnatal mouse ovaries (Mattison and Thorgeirsson, 1979, Mattison, 1980, Mattison and Nightingale, 1980).

Although a large volume of data has been reported for the ovotoxic properties of PAH over the past twenty years, comparatively little is known of the mechanisms by which PAH cause follicular destruction. Almost two decades ago, Mattison noted that the morphology of degenerating primordial oocytes in mice dosed with PAH *in vivo* resembled that which occurs during prenatal degeneration of the female germ line (Mattison, 1980). Based on the points already discussed, it is probable that apoptosis is the mechanism by which follicle loss occurs in response to PAH exposure (Tilly and

Perez, 1997). Nevertheless, it remains uncertain how PAH trigger apoptosis in oocytes. Several lines of evidence have indicated that metabolic conversion of PAH to reactive intermediates may be an important step in the ovotoxic response. In support of this hypothesis, a positive correlation between ovarian metabolic activity (activity of aryl hydrocarbon hydroxylase or AHH, an enzyme encoded by the P4501A1 gene) and ovotoxicity of PAH in several strains of mice has been reported (Mattison and Thorgeirsson, 1979, Mattison and Nightingale, 1980). Based on these data it has been proposed that free radical-like species, such as the diol-epoxides generated by AHH-catalyzed PAH metabolism, damage DNA and other intracellular components, events that culminate in cellular dissolution. However, significant levels of oocyte destruction occur in mice which possess relatively low levels of ovarian AHH activity (Mattison and Nightingale, 1980), suggesting that metabolism of the PAH to a reactive intermediate(s) is not the only mechanism involved in ovotoxicity. Along these lines, data derived from studies using compounds which are thought to inhibit PAH metabolism, such as a-naphthoflavone (ANF), have been put forth to support the concept that metabolic conversion of the parent PAH compound to reactive intermediates is required for ovotoxicity. For example, animals treated with PAH in the presence of ANF, *in vivo,* do not exhibit oocyte destruction (Mattison and Thorgeirsson, 1979). However, interpretation of these data requires a great deal of caution as more recent studies have shown that ANF and related flavone compounds function as potent antagonists of the cytoplasmic binding protein for PAH, the aryl hydrocarbon receptor (AhR) (Lu *et al.,* 1995, Hankinson, 1995). Therefore, the ovotoxic effects of PAH may be linked to their specific interaction with the AhR and the subsequent regulation of genes responsive to the ligand-activated AhR, in addition to/in lieu of metabolism of PAH to reactive intermediates that perturb cellular homeostasis. Future studies to investigate this possibility, with apoptosis-regulatory genes as candidate targets for PAH/AhR-regulated transcription, will be of interest since many cell death gene knockout mice now exist to confirm or refute this hypothesis (see sections 5.2.1 and 5.2.2).

5.3.3 Vinylcyclohexene (VCH)

This third example of ovarian toxicity is interesting because VCH and its metabolites are, from all accounts, relatively specific gonadal toxicants (Hoyer and Sipes, 1996). The chemical synthesis of tyres, insecticides, flame retardants and plasticizers releases by-products into the environment such as 4-vinyl-1-cyclohexene (VCH) and its epoxide metabolites, 4-vinyl-1-cyclohexene 1,2-epoxide, 4-vinyl-1-cyclohexene 7,8-epoxide and 4-vinyl-1-cyclohexene diepoxide (VCD) (Hoyer and Sipes, 1996). Daily intraperitoneal injection of mice with VCH for 30 days has been reported to destroy 85% of the oocytes contained within the smallest ovarian follicles (primordial, primary), whereas similar treatment of rats did not result in any apparent toxic effects within the ovary (Hooser *et al.,* 1993, 1994). Three epoxide metabolites of VCH were also tested and were found to

be up to 18-fold more potent than VCH in their ability to destroy small ovarian follicles in mice. Moreover, unlike VCH, these epoxide metabolites also caused oocyte loss in rats (Smith *et al.,* 1990b). Since epoxide metabolites were found to be more ovotoxic than the parent compound (VCH), it has been proposed that the conversion of VCH to the diepoxide form (VCD) is required for this compound to cause germ cell destruction. Data from *in vitro* studies suggest that the greater susceptibility of mouse ovaries, as compared to rat ovaries, to VCH is due to a species-related difference in the rate of epoxide formation in the liver (Smith *et al.,* 1990a). Mouse hepatic microsomes incubated with VCH form VCH-1, 2-epoxide at a rate 6.8-fold greater than rat hepatic microsomes. Additionally, human liver microsomes convert VCH to the epoxide form at a rate half of that observed for rat hepatic microsomes (Smith and Sipes, 1991). Consequently, if the generation of epoxide metabolites is required for the ovotoxicity of VCH, the rat probably offers a more appropriate experimental model to predict the effects of VCH in humans.

A study of the long-term effects of VCH exposure on the ovary have revealed that treatment of mice with VCH for 30 days results in markedly reduced ovarian and uterine weights, premature ovarian failure, increased circulating levels of follicle-stimulating hormone (FSH) and pre-neoplastic changes within 360 days of the start of VCH treatment, as compared to control animals (Hooser *et al.,* 1994). In contrast to the irreversible toxic effects of VCH on the ovary, however, male mice treated in an identical fashion displayed decreased testicular weight and a loss of sperm production that were rapidly reversed following cessation of VCH exposure (Hooser *et al.,* 1995). To uncover the possible mechanisms underlying VCD-induced ovarian damage, Springer *et al.* (1996a,b) conducted a parallel set of investigations in rats to address the possibility that apoptosis and cell-death regulatory genes, respectively, were components of primordial and primary follicle loss. Following a daily dosing regimen for up to 30 days, morphological assessments of oocyte and granulosa cell health status confirmed the occurrence of apoptosis as a time-dependent response to VCD treatment (Springer *et al.,* 1996a). Moreover, increased expression of the cell-death susceptibility gene, *bax,* was identified in primordial and primary follicles following VCD exposure at a time preceding any evidence of follicular cell apoptosis or follicle loss (Springer *et al.,* 1996b). These findings support the hypothesis that apoptosis in the ovary, occurring naturally or driven by toxicant exposure, involves a Bax-mediated pathway of execution (see Table 5.1).

5.3.4 Anti-cancer drugs

Many classes of cancer chemotherapeutic agents damage DNA leading to cell death, and reproductive tissues are highly vulnerable to unfortunate side-effects of such toxicants. Girls and women treated with these chemicals to combat cancer often show fertility problems and premature ovarian failure at some point post-therapy (Horning *et al.,* 1981,

Waxman, 1983, Familiari *et al.,* 1993). In men faced with the long-term consequences of cancer treatment, fertility can be spared by collection and cryopreservation of sperm prior to the commencement of therapy for use in subsequent assisted reproductive technologies (Meirow and Shenker, 1995). However, a similar course of action is essentially out of reach for women since oocytes harvested for cryopreservation have extremely poor rates of post-thaw fertilization and embryo development (Trounson and Bongso, 1996). Recent efforts to show a recovery of oestrous cyclicity in animal models subjected to transplant of cryopreserved ovarian autografts offer promise for the future (Baird *et al.,* 1999). However, there currently exist no clinically-proven interventions that can be used to protect the ovaries, *in vivo,* from the ravages of chemotherapy.

Unfortunately, little is known of the mechanisms underlying this paradigm of clinically-induced ovarian damage. Importantly, however, a recent series of investigations using female mice has established that oocytes respond to at least one widely-prescribed antineoplastic agent, doxorubicin, by undergoing apoptosis (Perez *et al.,* 1997). These findings provide the first insights into how cancer drugs may cause infertility, and raise the possibility that specific manipulation of apoptosis-regulatory molecules in oocytes may one day yield new approaches to combat premature ovarian failure in female cancer patients (Reynolds, 1999). In support of this concept, pharmacologic suppression of ceramide function or caspase activity, or *bax* deficiency through gene knockout, in murine oocytes prevents the apoptotic response to doxorubicin (Perez *et al.,* 1997). By comparison, p53 deficiency had no protective effect in oocytes exposed to chemotherapy, arguing the existence of cell type-specific requirements for various molecules associated with cell death regulation.

The involvement of caspases in ovotoxicity was suggested by findings that showed peptide inhibitors of caspases prevented oocyte fragmentation caused by doxorubicin treatment (Perez *et al.,* 1997). Follow-up studies showed that in caspase-2-deficient female mice, oocytes were resistant to doxorubicin-induced death (Bergeron *et al.,* 1998). At present, there are no reported studies to confirm the role of ceramide in oocyte loss as deduced from the pharmacologic inhibition studies with sphingosine-1-phosphate (Perez *et al.,* 1997). It should be stressed, however, that Perez *et al.* (1997) identified hydrolytic pathways (i.e. sphingomyelinases) as opposed to synthetic pathways (ceramide synthase) as the source of ceramide that may be produced in oocytes exposed to doxorubicin. Therefore, future studies of the ovarian response of acid sphingomyelinase-deficient female mice (Horinouchi *et al.,* 1995) to antineoplastic drugs will certainly be of interest.

5.4 SUMMARY

With the growing amount of literature pointing to apoptosis as a principal effector mechanism underlying ovarian toxicity (and probably toxicity in non-gonadal tissues

associated with female reproductive function as well), future efforts should continue to focus on elucidation of the intracellular events set in motion by xenobiotics in ovarian germ cells and somatic cells that lead to their untimely demise. Elaboration of the molecular framework through which specific chemicals wreak havoc in the ovary may one day lead to the development of new technologies to protect the precious reserve of female germ cells from premature death following toxicant exposure. One major consideration that must be met is designing approaches that specifically target ovarian cells, and in particular the germ line, for defense against pathologic insults. Although much work is needed to accomplish these goals, the recent identification of specific cell death genes being required for the ovotoxic response to chemotherapy (Perez *et al.,* 1997, Bergeron *et al.,* 1998), coupled with findings that oocyte-specific expression of protective molecules such as Bcl-2 (Morita *et al.,* 1999b) can be achieved, provide strong impetus to continue studies on understanding the impact of manipulating apoptosis in the context of female reprotoxicology.

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CHAPTER 6

Modulation of Apoptosis in Renal Toxicity

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6.1 Introduction
6.2 Apoptosis in the kidney
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6.1 INTRODUCTION

Apoptosis is a highly regulated mechanism of cell death that plays a role in pathogenic organ responses following toxicant exposure (see Chapter 1). There are many toxicants that induce apoptosis in kidney or renal cells although the relationship between aberrant triggering of the process by toxicants and renal toxicity remains to be defined. Perhaps the relatively recent interest in apoptosis as a mechanism of toxicant-induced renal damage can be correlated with the need to examine the more subtle effects of low level environmental exposures, minimizing extrapolation from higher doses. This chapter outlines identification of apoptosis in the kidney then explores its role and regulation in normal kidney development and function. Next, several renal toxicants are described followed by a discussion of the molecular mechanisms that may underpin their mode of action.

6.2 APOPTOSIS IN THE KIDNEY

Apoptosis is a term first introduced in the 1970s to define the morphology of dying cells (Kerr *et al.,* 1972) although Glucksmann and colleagues described the same morphologic alterations in the kidney during the early 1950s (Glucksmann, 1951). Apoptosis during kidney development and after tubular damage permits proper nephron formation and tubular repair, respectively.

6.2.1 Renal apoptosis: morphology and detection

As with other tissues and organs, the diagnosis of kidney apoptosis is not solely by morphology, but also by the frequency, distribution and timecourse of occurrence. Also, apoptotic bodies in the kidney and elsewhere typically undergo apoptotic necrosis (or secondary necrosis; Chapters 1 and 10) within the phagolysosomes of the engulfing cell (Figure 6.1). In contrast, cells not engulfed will proceed to a necrotic phase in the tissue and cellular contents will spill into the extracellular space, often inciting inflammation.

Histopathologic examination of fixed cells or tissue sections is required for a definitive diagnosis of apoptosis in renal tissue or renal cell culture. As described in Chapter 10, various stains make nuclear fragmentation readily apparent in fixed tissues or fixed cells (McGahon *et al.,* 1995) and an acridine orange/ethidium bromide combination is frequently used in cell culture to distinguish early, late or necrotic kidney cells (Darzynkiewicz *et al.,* 1994). Similarly, Hoechst 33258 or DAPI will detect chromatin fragmentation and/or condensation. DNA fragmentation can be detected using agarose gel electrophoresis, the JAM assay, *in situ* end labelling or terminal deoxytidyl transferase-mediated dUTP nick-end labelling (ISEL or TUNEL) or anti-histone antibodies (which detect histone bound DNA fragments (Walker *et al.,* 1993, Wijsman *et al.,* 1993) (Chapter 10). In kidney, there is usually no inflammatory response mounted against apoptotic

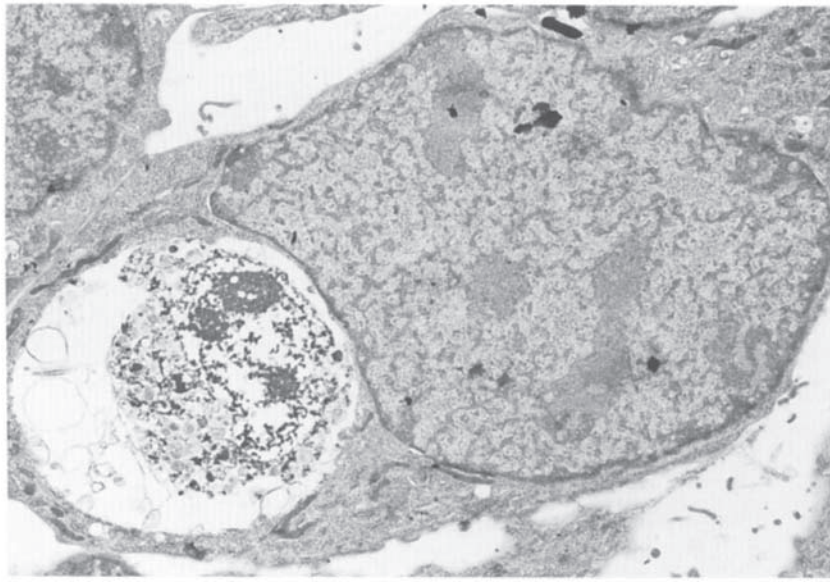

Figure 6.1 Cell engulfment and secondary necrosis. Primary cultures of rat proximal tubules were exposed to TNFα *in vitro* and processed for electron microscopy. A proximal epithelial cell is shown in the cytoplasm of a neighbouring cell after phagocytosis. The engulfed cell shows morphologic evidence of secondary necrosis. Reproduced with permission from *Toxicologic Pathology,* 1998.

bodies since they are quickly ingested by neighbouring cells or shed into the tubular lumen. However, as mentioned earlier, a combination of apoptosis/oncosis and necrosis can occur *in vivo* which will attract inflammatory cells to the area. Recognition and engulfment of apoptotic cells and cell fragments is associated with externalization of phosphatidylserine (PS) from the inner plasma membrane to the cell surface (Stuart *et al.,* 1998). There are numerous techniques available to detect many of the biochemical changes that occur during apoptosis and some of these are covered in Chapter 10 of this volume. Many of these techniques are also useful in mechanistic studies. The biochemical tests chosen should be used to complement, but not to replace morphologic examination.

6.2.2 Apoptosis during kidney development

The ureteric bud is an outgrowth of the wolfian ducts that secretes a variety of factors which protect the mesenchymal cells from apoptosis whereas diffusion-limited basolateral factors trigger the conversion of mesenchyme into epithelium (Barasch *et al.,* 1996) (reviewed in Davies, 1993, Lechner and Dressier, 1997). Generally, apoptosis is the fate of the uninduced, undifferentiated mesenchymal cells and cells residing in the walls of tubular structures (Coles *et al.,* 1993) such as type B intercalated cells of the medullary

TABLE 6.1
Roles of apoptosis in renal disease

Disease	Role of apoptosis in pathogenesis	Sites of apoptosis	References
Congenital dysplastic kidneys	Contributes to renal malformation	Mesenchymal cells surrounding the dysplastic tubules	Kreidberg *et al.*, 1993, Winyard *et al.*, 1996
Diabetic nephropathy	May cause atrophy of tubular epithelium	Tubular epithelium	Ortiz-Arduan *et al.*, 1996
Glomerulonephritis	Resolution of the disease; progression to glomerular sclerosis	Glomeruli	Takemura *et al.*, 1995, Shimizu *et al.*, 1996a,b
Glomerular sclerosis	Correlates with loss of renal function	Tubular epithelium; mesangial cells, interstitial cells, endothelial cells	Shimizu *et al.*, 1996
Hemolytic uremic syndrome	Destruction of microvasculature	Endothelial cells Tubular epithelium	Uchida *et al.*, 1997 Williams *et al.*, 1997
HIV nephropathy	Increased apoptosis in tubular epithelium leads to decreased tubular function	Tubular epithelium	Bodi *et al.*, 1995
Obstructive uropathy and hydronephrosis	Contributes to tubular atrophy and renal tissue loss	Tubular epithelium Interstitial cells	Chevalier *et al.*, 1996 Cummings *et al.*, 1996, Truong *et al.*, 1996
Polycystic kidney disease	Loss of normal renal tissue; may account for loss of renal function	Interstitial cells Cystic epithelium Proximal tubular epithelium	Lanoix *et al.*, 1996 Winyard *et al.*, 1996 Woo, 1995
Renal cancer	May modify tumour growth; susceptibility may also determine response to chemotherapeutic agents	Tumour specific	Bardeesy *et al.*, 1995, Chandler *et al.*, 1994, Hofmockel *et al.*, 1996, Paraf *et al.*, 1995, Tannapfel *et al.*, 1997

collecting duct (Kim *et al.,* 1996a). Without the inducing effect of the ureteric bud, the entire nephrogenic mesenchyme undergoes widespread apoptosis (Koseki, 1993). The thin ascending limb of the loop of henle is derived from the thick ascending limb by apoptotic deletion of the thick ascending limb followed by cell transformation whereas, the type A intercalated cells are removed by extrusion from the epithelium (Kim *et al.,* 1996b). There is a distinct temporal pattern of apoptotic deletion during rat kidney development. Approximately 2.7% of cells within the nephrogenic zone are apoptotic during the embryonic period which declines to a basal level of 0.15% by the fourteenth

postnatal day (Coles *et al.,* 1993). A second peak of apoptosis occurs in the medullary papilla at postnatal days 6–7 when the percentage of apoptotic cells reached 3.2%. Peak apoptosis in the medullary papilla correlates temporally with deletion of the thick ascending limb cell during the formation of the ascending thin limb of the loop of Henle (Kim *et al.,* 1996b). The predicted effect of renal toxicant exposure *in utero* will depend on the cellular events during that developmental period and whether the toxicant induces or inhibits apoptosis. An inducer of apoptosis may kill cell populations that are required for normal tubular function whereas an apoptosis inhibitor may result in malformed tubules of cells with a poorly differentiated phenotype. Many of the renal diseases associated with altered apoptosis are summarized in Table 6.1.

6.3 MOLECULAR CONTROL OF RENAL APOPTOSIS

Tremendous progress has been made in understanding the intracellular mechanisms of apoptosis and these pathways are reviewed in detail in Chapter 1. However, certain factors have particular relevance for the kidney; these are discussed below.

6.3.1 Tumour necrosis factor α (TNFα) and Fas

The tumour necrosis factor (TNF) receptor family is one of the best characterized of the receptor-mediated cell death pathways (see Chapters 1 and 2). TNFα and the Fas ligand Fas-L) are the cytokine ligands of the TNF superfamily which, upon binding to their receptors, engage the apoptotic pathway via a region of intracellular homology named the 'death domain' (Vandevoorde *et al.,* 1997). Neither receptor has intrinsic catalytic activity, but through the death domain, they induce a cascade of protein interactions that result in activation of apoptotic executioners such as caspases (Muzio *et al.,* 1998, for review see Baker and Reddy, 1996) (Chapter 2). The regulation of Fas and TNFα pathways may also involve the activation of pro-apoptotic protein kinases such as p38 (Goillot *et al.,* 1997) and Jun N terminal Kinase (JNK), sphingomyelinase activation and generation of sphin-gomyelin metabolites such as ceramide (Tepper *et al.,* 1995, Sweeney *et al.,* 1996, Ichijo *et al.,* 1997). Tumour necrosis factor (TNF) has been shown to cause apoptosis in numerous renal cells in culture including mouse mesangial cells, LLCPK, and mouse proximal tubular cells (Pelletier *et al.,* 1991, Liu *et al.,* 1996, Ortiz-Arduan *et al.,* 1996). Indeed, decreased susceptibility of renal tumour cells to TNF-induced apoptosis is also an important mechanism of chemotherapeutic resistance (Woo *et al.,* 1996). Fas-L expressed on lymphocytes triggers apoptosis in renal cell carcinoma cell lines and murine mesangial and tubular epithelial cells (Ortiz-Arduan *et al.,* 1996, Tomita *et al.,* 1996). The TNF family includes the cell surface receptor CD95 (also called Fas or APO-1), nerve growth factor receptor, CD40 and CD30 (120). CD95 differs from the TNF receptor in that normal human renal cells do not express the CD95 receptor (Kato *et al.,* 1997).

6.3.2 Granzyme B

Granzyme B is a serine esterase produced by cytotoxic T lymphocytes and natural killer cells. In most cases, granzyme B enters the targeted cell via perforin mediated pore formation, but it can also cross the cellular membrane independent of perforin (Shi *et al.,* 1997). Within the targeted cell, granzyme B causes proteolysis of intracellular components and apoptosis (Williams and Henkart, 1994). Studies on renal cells in culture indicate that expression of the nuclear kinase Weel can protect against granzyme B and perforin induced apoptosis (Chen *et al.,* 1995b). Interestingly, increased levels of granzyme B and perforin correlate with acute rejection of renal transplants (Kummer *et al.,* 1995, Sharma *et al.,* 1996, Strehlau *et al.,* 1997, Suthanthiran, 1997).

6.3.3 Cysteine aspartic serine proteases (caspases)

The caspases are a family of cysteine aspartic serine proteases (reviewed in Cohen, 1997) decribed in detail in Chapter 2. Two members of the caspase family, caspase 1 (interleukin 1 converting enzyme; ICE) and Nedd have been localized within the murine foetal kidney (Kumar *et al.,* 1994) and caspase 3 (CPP32) has been localized within the human renal tubular epithelium (Krajewski *et al.,* 1996). The essential role of caspases in apoptosis appears to be in the execution phase although a common feature of the identified caspase substrates (see Chapter 2) is that they all play a vital role in maintenance of cellular homeostasis and function. Recent studies suggest that numerous signal transduction proteins are caspase substrates and that their cleavage contributes to cell death (Krajewski *et al.,* 1996, Widmann *et al.,* 1998a, b). Mitogen activated protein kinase kinase kinase (MEKK) was shown to be cleaved to a 91 kDa fragment that activates caspases. In addition, Cbl, Cblb, Raf, Focal adhesion kinase and AKT appear to be substrates for caspases. Additional studies are certain to identify many additional caspase substrates in the coming years.

6.3.4 The Bcl-2 family

The Bcl-2 family of proteins is a closely associated group of intracellular pro- and antiapoptotic regulators. Six members of the family have been specifically mapped to various locations within the kidney; Bcl-2, Bcl-XL, Bfl-1, Bax, Bak, MCL-1 and BAD (Eguchi *et al.,* 1992, Gonzalez-Garcia *et al.,* 1994, McDonnell *et al.,* 1994, D'Sa *et al.,* 1996, Krajewski *et al.,* 1996, Sorenson *et al.,* 1996). During renal development, Bcl-2 expression has been reported in the murine ureteric bud and tubular structures (Novack and Korsmeyer, 1994) and in induced human mesenchymal cells (McDonnell *et al.,* 1994). Intracellularly, Bcl-2 is localized with mitochondrial, endoplasmic reticulum and nuclear membranes (for review of the Bcl-2 family see Korsmeyer *et al.,* 1995, Kroemer, 1997). Bcl-2 also has unique distribution during mitosis; it appears in early prophase or late G2 and rapidly

disappears at telophase. There appeared to be a specific concentration of Bcl-2 at the margins of condensed chromatin (Willingham and Bhalla, 1994).

The anti-apoptotic proteins of the Bcl-2 family are uniquely associated with regulation of apoptosis in the kidney. During murine development, a lack of Bcl-2 results in increased apoptosis and cystogenesis (Sorenson *et al.,* 1995, 1996). In contrast, overexpression of Bcl-2 may contribute to the development of renal neoplasia. The mechanism(s) by which Bcl-2 family members function are presently unclear. There are numerous biological effects of Bcl-2 on intact cells and as a result, various mechanisms have been proposed to explain how the Bcl-2 family members functionally regulate apoptosis. The proposed mechanisms of anti-apoptotic function include protection of membranes from oxidant damage, antagonism of pro-apoptotic proteins such as Bax and protection of organelle membranes by regulating permeability via ion channel formation (Fabisiak *et al.,* 1997, Minn *et al.,* 1997, Schendel *et al.,* 1997). In addition, serine phosphorylation also appears to modulate the function of some Bcl-2 family members such as Bcl-2 and BAD (Haidar *et al.,* 1996, Zha *et al.,* 1996). In a general sense, renal epithelial cells are unique in their physiological relevance to many of the proposed mechanisms of Bcl-2 function since ion channels, response to oxidant stress, and pH regulation are normal functions carried out by renal epithelial cells *in vivo*.

6.3.5 p53 and WT-1

P53 is a zinc finger transcription factor which induces the transcription of a variety of proteins including p21, Gadd45, and Gadd153 (Jeong *et al.,* 1997a, b) which are involved in cell cycle arrest or DNA repair. High expression of p53 protein in murine renal development is associated with increased apoptosis and kidney failure in mice (Godley *et al.,* 1996). Similarly, the Wilms' tumour suppression gene (WT-1) is an important regulator of apoptosis in kidney (Englert *et al.,* 1995) and plays a critical role in renal development. Homozygous mutation of murine WT-1 is lethal and results in failure of the development of many organs including the kidney (Kreidberg *et al.,* 1993). Heterozygous mutations have been associated with formation of Wilms' tumour (Little *et al.,* 1992) and urogenital anomalies associated with Denys-Drash syndrome (Pelletier *et al.,* 1991). The mechanism by which WT-1 regulates apoptosis is unclear. However, in human kidney cells, WT-1 has been shown to suppress the synthesis of Bcl-2 and c-myc and to inhibit p53-induced apoptosis in rat kidney (Hewitt *et al.,* 1995, Maheswaran *et al.,* 1995).

6.3.6 Growth factors

Growth factors provide a survival signal that suppresses apoptosis in most cell populations; growth factor deprivation induces one of two responses, quiescence or apoptosis. Apoptosis in the metanephric mesenchyme is prevented by epidermal growth factor (EGF) and

basic fibroblast growth factor (bFGF). In the adult kidney, acceleration of renal repair following tubular damage can be achieved by exogenous administration of growth factors (Takeda and Endou, 1996). Classically, growth factors were thought to contribute to renal repair by activating cell signalling pathways that induce cell proliferation. However, it is now apparent that growth factors also serve to inhibit apoptosis in the kidney both during development and in renal repair of adult kidneys independent of proliferative effects. The response of the cell to growth factors may depend upon the state of the cell since EGF was shown to abrogate apoptosis of renal epithelial cells without affecting mitotic index (Coles *et al.,* 1993). Similarly, exogenous insulin growth factor (IGF-1), EGF or hepatocyte growth factor (HGF) administered in the early phase post ischemia accelerates renal tubular recovery (Humes *et al.,* 1989). The precise roles of HGF and IGF-1 in apoptosis in the kidney is unclear. However, IGF-1 was shown to prevent apoptosis in serum-deprived PC12 cells, correlating with an increase in *bcl-x* mRNA and protein (Parrizas and LeRoith, 1997). After ischemic renal injury, IGF-1 expression is localized to the regenerative zones of the renal tubule (Lake and Humes, 1994). In addition, the therapeutic effects of hepatocyte growth factor associated with suppression of apoptosis have been demonstrated in drug-induced nephrotoxicity (Woo *et al.,* 1996).

6.3.7 Extracellular matrix interactions

Proper interaction with the extracellular matrix is necessary to prevent spontaneous apoptosis in most epithelial cells. Anoikis (pronounced an-o-É-kis) is a special term used to describe the entry of epithelial or endothelial cells into apoptosis when they lose contact with the extracellular matrix (Frisch, 1994). Activation of apoptosis through detachment is especially important *in vivo* because it insures that detached cells are unable to proliferate or survive in an inappropriate location after detachment inhibiting metastasis. The intracellular signals linking the extracellular matrix to cell response pathways include phosphoinositide 3-OH (PI3) kinase, Ras, focal adhesion kinase and the mitogen activated protein kinase pathways (Frisch and Dolter, 1995, Khan *et al.,* 1995, Cardone *et al.,* 1997). The intracellular signals regulated by extracellular matrix interaction at the cell surface appear to be linked through the integrin family of cell surface proteins (Saelman *et al.,* 1995, Strater *et al.,* 1997). Disrupting the ability alpha 2 beta 1 integrin to bind to the type I and IV collagen caused increased apoptosis in the Madin-Darby canine kidney (MDCK) (Saelman *et al.,* 1995) and inhibition of focal adhesion kinase, a cytoplasmic tyrosine kinase which acts as an integrin transducer, induces apoptosis (Frisch *et al.,* 1996). Cell matrix regulated signal pathways may also mediate the function of anti-apoptotic and pro-apoptotic proteins such as Bcl-2 (Frisch *et al.,* 1996, Zha *et al.,* 1996). Phosphorylation of BAD (a pro-apoptotic member of the Bcl-2 family) by AKT in a PI3-kinase dependent pathway was shown to inactivate BAD and promote cell survival (del Peso *et al.,* 1997). Similarly, cell-to-cell interactions that sensitize cells to anoikis have been shown to down-regulate *bcl-2* mRNA and protein (Frisch *et al.,* 1996).

Alternatively, the cell's connection to the extracellular matrix may influence apoptosis by directly influencing cell spreading. The degree of cell spreading of endothelial cells was key to regulating apoptosis in endothelial cells (Chen *et al.,* 1997). Since the cell shape is dictated by the number of attachments the cell has with the basement membrane, an increase in the area that a cell can occupy will allow the cells to spread and suppress cell death in the remaining cells. An example of a situation in which the area for cell spreading increases can be found in acute tubular necrosis when cells are lost from the basement membrane due to tubular damage.

6.3.8 Clusterin

Clusterin (also termed sulphated glycoprotein-2 (SGP2), testosterone repressed prostate message-2 (TRPM-2) or apoplipoprotein) may be linked mechanistically to apoptosis in the kidney due to its role in aggregation of renal epithelial cells (French *et al.,* 1994). However, although the association is quite clear, the precise regulatory role of clusterin in renal apoptosis is still unknown. The increase in clusterin expression may act as a protection mechanism in the damaged tubules, promoting cell interaction and cell signalling in the surviving cells.

6.3.9 Protein phosphorylation

Protein phosphorylation is a protein modification that regulates the activity of a number of cellular factors involved in apoptosis. The active state of the substrate protein will ultimately determine the effect of phosphorylation on protein activity and the cellular response to the modification. Multiple phosphorylation based signal transduction cascades such as the mitogen activated protein kinase family (MAPK) and stress activated protein kinases (SAPK) appear to be involved in mediating apoptosis in kidney. A pattern of kinase activation (p38, extracellular regulatory kinase (ERK) and JNK) together with stress response genes (c-jun, ATF-2) correlates with apoptosis in kidney after ischemia reperfusion (Xia *et al.,* 1995). The importance of this correlation is underscored by the improvement in renal function and improved outcome of ischemic renal failure afforded by down-regulation of MAPK (Di Mari, 1997a). The precise role of kinase activation in ischemia reperfusion is still unclear since these kinases can regulate proliferative and apoptotic pathways.

Protein phosphatases, PP1 and PP2A, appear to regulate proliferation in early embryonic kidney with PP1 and PP2A expression highest in E15 kidney (Svennilson *et al.,* 1995). In neurons, a balance between growth factor activated ERK and p38/JNK is proposed to be the switch for apoptosis or cell proliferation (Woolf *et al.,* 1995). A similar balance in kinase and phosphatase activity may be regulatory in renal cells. Kinase signalling pathways are especially relevant to toxicants since they are the major intracellular transducers for endogenous molecules and thus could be employed by a number of exogenous chemicals.

6.4 TOXICANT-INDUCED RENAL APOPTOSIS

A growing list of toxicants and natural toxins are associated with alterations in renal apoptosis (Tables 6.2 and 6.3), Although various heavy metals, pharmacologic agents and naturally occurring toxins cause apoptosis in the kidney, the mechanisms and molecular

TABLE 6.2
Pro-apoptotic toxins and toxicants

Toxin/Toxicant	Class	Source(s)
Alternaria alternata lycopersici toxin	Mycotoxin	Wang *et al.*, 1996 Winter *et al.*, 1996
Cadmium	Heavy metal	Hamada *et al.*, 1991, Hamada *et al.*, 1994
Ceramide	Sphingolipid metabolite	Jarvis *et al.*, 1994
Cisplatin	Antineoplastic drug	Leiberthal *et al.*, 1996, Takeda *et al.*, 1996
Curcumin	Dietary pigment/spice	Jiang *et al.*, 1996
Cyclophosphamide	Antineoplastic drug	Cha *et al.*, 1996
Cyclosporin A	Immunosuppressant	Cha *et al.*, 1996
Dexamethasone	Antineoplastic drug	Cha *et al.*, 1996
Doxorubicin	Antineoplastic drug	Zhang *et al.*, 1996
Dithiothreitol	Reducing agent	Van de Water *et al.*, 1996a
Fumonisins	Mycotoxin	Tolleson *et al.*, 1996, Voss *et al.*, 1996, Wang *et al.*, 1996
Gliotoxin	Mycotoxin	Waring *et al.*, 1994
Hygromycin	Antibiotic	Chen *et al.*, 1995a
Melphalan	Antineoplastic drug	Cha *et al.*, 1996
Mercuric chloride	Heavy metal	Duncan-Achanzar *et al.*, 1996, Nath *et al.*, 1996
Metolazone	Thiazide diuretic	Loffing *et al.*, 1996
Microcystin LR	Cyanobacterial toxin	Khan *et al.*, 1995, MacKintosh *et al.*, 1990, MacKintosh *et al.*, 1995
Ochratoxin A	Mycotoxin	Seegers *et al.*, 1994
Okadaic Acid	Poly ether fatty acid	Davis *et al.*, 1994, 1996
Ricin	Ribosome inactivating protein	Williams *et al.*, 1997
s-(1,2 dichlorovinyl)-L-cysteine	Reducing agent	Van de Water *et al.*, 1996a
Verocytotoxin	*E. coli* enterotoxin	Williams *et al.*, 1997

TABLE 6.3
Pro and anti-apoptotic viral proteins

Virus/Viral protein	Source(s)
Adenovirus E1A protein	Frisch *et al.*, 1995, Mymryk *et al.*, 1994, Rao *et al.*, 1992, Sabbatini *et al.*, 1995a
Adenovirus E1A12S protein	Quinlan *et al.*, 1993
Adenovirus E1B protein	Chiou *et al.*, 1994b, Sabbatini *et al.*, 1995b
Cowpox (crmA negative)	Ray *et al.*, 1996
Dearing reovirus (type 3)	Rodgers *et al.*, 1997
Epstein Barr virus BHRF 1 protein	Theodorakis *et al.*, 1996
Human papilloma virus E6 protein	Thomas *et al.*, 1996
Influenza A, B and C	Hechtfischer *et al.*, 1997, Hinshaw *et al.*, 1994
Lang reovirus (Type 1)	Rodgers *et al.*, 1997
Sindbis virus	Levine *et al.*, 1993

pathways employed are diverse. Exposure to renal toxicants that affect apoptosis in kidney can result in altered renal function and contribute to or cause renal disease.

6.4.1 Natural toxins

Many natural toxins such as verocytotoxin and ricin are potent inducers of apoptosis. Verocytotoxin (produced by *E.coli*) induces apoptosis in renal cells which is potentiated by tumour necrosis factor beta (TNFβ) (Uchida *et al.,* 1997). TNFβ increases the number of verocytotoxin receptors on the endothelial cell surface and has been linked to hemolytic uremic syndrome (Richardson *et al.,* 1988, van Setten *et al.,* 1997). Ricin (from the castor bean) has been reported to induce apoptosis of Vero cells (derived from green monkey kidney) by inhibiting ribosomal protein synthesis (Williams *et al.,* 1997). Recently, diisopropyl fluorophosphate, a general serine protease inhibitor, has been shown to inhibit ricin-induced DNA fragmentation and cell death of MDCK cells (Oda *et al.,* 1998). In those studies, protein synthesis was not inhibited suggesting that the pathway of ricin-induced cell death may be independent of the pathway of protein synthesis inhibition.

Toxins such as *Alternaria alternata lycopersici* (AAL) and fumonisin are sphingonine analogue mycotoxins which induce apoptosis in the kidney. These toxins contaminate food and water supplies, increasing their potential for renal exposure (Dutton, 1996, Wang *et al.,* 1996). AAL and fumonisin induce apoptosis in tubular epithelial cells; this is most prevalent in the outer medulla and is associated with development of hydronephrosis (Lin *et al.,* 1995, Tolleson *et al., 1996,* Sharma *et al.,* 1997). In rats and

rabbits, the kidney is the most sensitive target organ to these toxins (Bucci *et al.,* 1996). The mechanism of AAL or fumonisin-induced apoptosis is not fully understood, but they cause disruption of sphingolipid metabolism (reviewed in Pena *et al.,* 1997) by inhibition of ceramide synthase and/or inhibiting n-acyl transferase (Wang *et al.,* 1991, Norred *et al.,* 1992, Merrill *et al.,* 1993). Exogenous ceramide triggers apoptosis in numerous cell types (Jarvis *et al.,* 1994, Tepper *et al.,* 1995) and since sphingolipids are found in many foods such as beef and cheese, intestinal metabolism of sphingolipids to ceramides could potentially supply the kidney with a dietary source of ceramide (Schmelz *et al.,* 1994). Fumonisin B1 also appears to increase TNFα production by macrophages which may contribute to fumonisin B1-induced apoptosis *in vivo* (Dugyala *et al.,* 1998).

The natural toxins, ochratoxin A, microcystin LR and okadaic acid are important inducers of apoptosis of renal cells. Ochratoxin A is a product of strains of Aspergillus and Penicillium fungi (Veldman *et al.,* 1992) that induces apoptosis in kidney cells (Seegers *et al.,* 1994) via its affect on mitochondrial respiration and total cellular oxygen consumption (Veldman *et al.,* 1992, Seegers *et al.,* 1994). Unlike ochratoxin, microcystin LR and okadaic acid are protein phosphatase inhibitors that induce apoptosis of renal epithelial cells *in vitro* (MacKintosh *et al.,* 1990, Davis *et al.,* 1994, Khan *et al.,* 1995). The regulation of apoptosis by phosphatase inhibitors appears to involve complex kinase signalling pathways. Interestingly, H-ras oncogene expression increases susceptibility to okadaic acid-induced apoptosis (Davis *et al.,* 1996). Other toxins have been specifically associated with apoptosis of transformed renal cells. Curcumin, a yellow dietary pigment found in the spice, tumeric, has been shown to cause apoptosis of renal cancer cells by inhibiting protein kinase C (Jiang *et al.,* 1996) and the mycotoxin, gliotoxin, has been found to cause apoptosis in Wilm's tumour cells (Waring *et al.,* 1994).

Numerous viral toxins have been shown to mediate renal apoptosis (Table 6.3). Viruses or viral proteins which stimulate or inhibit renal apoptosis include the SV40T antigen, adenovirus E1A protein, influenza A virus, influenza B virus, cowpox viruses which lack the crm A gene, sindbis virus, and reovirus (Rao *et al.,* 1992, Levine *et al.,* 1993, Hinshaw *et al.,* 1994, Mymryk *et al.,* 1994, Sabbatini *et al.,* 1995b, *Ray et al.,* 1996, Martel *et al.,* 1997, Rodgers *et al.,* 1997). Viral inhibitors of apoptosis in the kidney include Epstein barr virus BHRF 1 protein, human papilloma virus 18E6 protein, human immunodeficiency virus TAT gene product, adenovirus E1A12S protein and adenovirus E1B 19-kDa protein (Quinlan, 1993, Sabbatini *et al.,* 1995a, Subramanian *et al.,* 1995, Theodorakis *et al.,* 1996, Thomas *et al.,* 1996, Yin *et al.,* 1997). The mechanisms involved in viral associated apoptosis vary. Caspase activation appears to accompany CrmA/SPI-2 mutants of cowpox and rabbitpox viruses (Macen *et al.,* 1998, Nava *et al.,* 1998). Interestingly, the pathogenesis of HIV associated nephropathy, a progressive glomerular and tubular disease that is common in AIDS patients, involves apoptosis directly mediated by HIV-1 expression (Bruggeman *et al.,* 1997).

6.4.2 Metals

The kidney is the principle target organ of toxicity for many metals. Many of the toxic effects of metals may be attributable to the induction of apoptosis in renal cells, but the molecular pathways are primarily unclear. For example, inorganic mercuric chloride induces apoptosis of porcine renal proximal tubule cells (LLCPK) although it appears to activate both pro-apoptotic and anti-apoptotic pathways. Exposure of LLCPK1 cells to $HgCl_2$ causes a rise in intracellular hydrogen peroxide level and it induces anti-apoptotic proteins such as Bcl-2 and Bcl-x (Duncan *et al.,* 1996, Nath *et al.,* 1996). In this case, elevation in anti-apoptotic Bcl-2 family members may be part of a generalized elevation of redox-sensitive genes in response to oxidative stress.

Metals such as iron induce apoptosis via an oxidative stress mechanism. Iron-induced apoptosis of mouse renal proximal tubular cells has been reported in the toxicity of iron-nitrilotriacetate. In these studies, iron appears to cause free radical generation (Kawabata *et al.,* 1997). Cadmium induces apoptosis of proximal convoluted tubular cells when administered to dogs or added to primary cultures of renal proximal tubules isolated from canine kidney (Hamada *et al.,* 1991, 1994). Normally, cadmium is rapidly bound to methallothionein (Cd-MT) in the liver and binding is generally considered to be protective to cells. However, it has been hypothesized that the Cd-MT complex may play a mechanistic role in renal apoptosis by increasing nuclear accumulation of Cd and inducing nuclear changes (Hamada *et al.,* 1996). Cd has been localized to the cytoplasm and nucleus of proximal renal epithelial cells and apoptosis may be protective in Cd toxicity since the apoptotic cells loaded with Cd-MT are shed into the urine providing a unique mechanism for cadmium excretion (Tanimoto *et al.,* 1993).

6.4.3 Antineoplastic agents and therapies

The toxicologic effects of several antineoplastic agent include the induction of apoptosis in renal epithelial cells. Cisplatin is one of the most well studied renal toxicants whose major cytotoxic effect is apoptosis of the proximal tubule cells. Cisplatin induces apoptosis at low concentrations, resulting in cell loss over the space of several days which appears to be mediated by reactive oxygen species generation (Lieberthal *et al.,* 1996). Other studies have implied that cisplatin-induced apoptosis is mediated by thromboxane A2 (Jariyawat *et al.,* 1997). Several microtubule damaging drugs such as paclitaxel, vincristin and vinblastine have been reported to activate protein kinase A and C-Jun-N-terminal kinase in response to microtubule damage, suggesting that distinct signal transduction cascades may be induced by cytoskeletal damage (Srivastava *et al.,* 1998, Wang *et al.,* 1998). Another group of nephrotoxicants causes apoptosis specifically in association with cytoskeletal disorganization in rat renal proximal tubules. Members of this group include S-(1,2dichlorovinyl) -L-cysteine (DCVC), cytochalasin D and dithiothreitol (Van

de Water *et al.,* 1996a). DCVC also results in increased intracellular calcium, mitochondrial damage and oxidative stress, all of which may also play a role in induction of apoptosis (Vamvakas *et al.,* 1990, Van de Water *et al.,* 1996b).

Exposure of kidney cells *in vitro* or *in vivo* to ionizing radiation induces apoptosis. Foetal kidneys exposed to radiation underwent extensive apoptosis, with the highest amount of apoptosis occurring within 24 hours of exposure. In the foetal kidney, the most sensitive cells are the rapidly dividing cells of the developing nephrons while those cells within the more mature nephrons suffered less damage (Gobe *et al.,* 1988). Adult mesangial cells are also susceptible to the apoptosis inducing effects of ionizing radiation (Cha *et al.,* 1996). Local delivery of interferon alpha was shown to increase the radiosensitivity of renal cell carcinoma (Syljuasen *et al.,* 1997). Gamma radiation does not have similar apoptotic inducing effects on the kidney or other radioresistant tissues such as heart, skeletal muscle, brain, liver, lung (Kitada *et al.,* 1996). However, in radiosensitive tissues, gamma radiation induces apoptosis and up-regulation of Bax protein.

6.5 MOLECULAR REGULATION OF TOXICANT-INDUCED APOPTOSIS

There are many diverse toxins and toxicants that induce renal apoptosis and these have been outlined above. However, information on the molecular mechanisms underlying this induction of cell death is incomplete. There are numerous instances in which TNF receptor ligands are elevated *in vivo,* increasing susceptibility of renal cells to apoptosis and renal damage. TNFα levels are increased in rat glomeruli during adriamycin or puromycin aminonucleoside-induced hydronephrosis (Gomez *et al.,* 1994). TNFα elevation may also potentiate the apoptotic effects of exogenous toxins or chemicals. Indeed, TNFα increases the apoptotic effects of verocytotoxin producing *E.coli* by increasing the number of verocytotoxin receptors on human renal endothelial cells (van Setten *et al.,* 1997). CD95 receptor mRNA expression is increased in diseased kidney and in human renal allografts undergoing acute rejection (Sharma *et al.,* 1996) and in cultured renal epithelial cells exposed to TNFα, *E.coli* lipopolysaccharide, interleukin 1-beta, or gamma interferon (Ortiz-Arduan *et al.,* 1996).

Glucose is an important molecular trigger of apoptosis of the renal cells. Thus, alterations in glucose metabolism that occur in diabetes or toxicant exposure may induce or increase renal susceptibility to apoptosis. The mechanism by which glucose modulates apoptosis appears to involve changes in the Bax: Bcl-2 ratios in murine tubular epithelial cells (Ortiz *et al.,* 1997). By decreasing the levels of Bcl-2 and increasing Bax levels, incubation with 25 mmol glucose engages the apoptotic process (Ortiz *et al.,* 1997).

Inhibition of PP1 and PP2A activity by the toxicant, okadaic acid, inhibited growth, nephron formation and induced apoptosis of developing nephrons. Similarly, okadaic acid-induced apoptosis of renal epithelial cells in culture (Davis *et al.,* 1994). Calcineurin, a calcium-dependent protein phosphatase appears to regulate calcium-induced apoptosis

in renal cells deprived of growth factors which is dependent on calcineurin's phosphatase activity (Shibasaki and McKeon, 1995). The inhibition of phosphatase activity by okadaic acid was accompanied by an increase in various kinase activities (Davis *et al.,* 1996). Thus the induction of apoptosis by phosphatase inhibitors may be due to the loss of phosphatase activity and the concomitant activation of kinases.

Tyrosine phosphorylation may link numerous seemingly independent induction pathways of renal apoptosis such as hypoxia, glucose and nitric oxide. Nitric oxide stimulates JNK activity in glomerular mesangial cells which was attenuated by genistein and N-acetylcysteine (DiMari *et al.,* 1997b). Similarly, D-glucose increases apoptosis and decreases replication in cultured metanephroi by perturbing phosphorylation of proteins that regulate perlecan (a mediator of epithelial mesenchymal interaction) (Kanwar *et al.,* 1996). In addition, chemical hypoxia (antimycin A and substrate deprivation) caused cell death and induced an increase in protein tyrosine kinase activity and protein tyrosine phosphorylation in LLCPK1 cells which was also inhibited by genistein. All of these cases support a role for protein phosphorylation as a common link in various mechanisms of apoptosis in renal cells.

6.6 SUMMARY

The results discussed in this chapter emphasize the importance of apoptosis in the kidney and in the potential functional consequences of toxicant or pharmacologic regulation of the process. The ongoing challenge is to study toxicants and novel pharmacologic agents to identify induction pathways that will be useful in drug design, preventative therapies and risk assessment. Since many toxicants and pharmacologic agents function during specific points in the cell cycle or have specific intracellular targets, they will provide a direct link to drug design and strategies that enhance or suppress apoptosis. We should look forward to seeing the use of these agents extended to therapeutic control of viral infection, cancer or developmental abnormalities.

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CHAPTER 7

Role of Apoptosis in Neuronal Toxicology

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7.1 INTRODUCTION

Cell death by apoptosis plays a fundamental role in the nervous system. It is essential for establishing neuronal networks during development since neurones initially are over-produced and the excess subsequently eliminated by apoptosis (reviewed in Oppenheim, 1991). Neurones require trophic support from several sources including their target cells and the Schwann cells that myelinate their processes (Snider, 1994) (Figure 7.1), and the blockade or removal of these signals induces cell death. The molecular mechanisms underlying the response of cells to such survival signals involves the Bcl-2 family of proteins.

Certain features of neurones such as their postmitotic phenotype and longevity make them susceptible to particular types of toxic insult. Despite this, there are few well documented examples of toxicants inducing neuronal cell death. This review will describe the occurrence and molecular mechanisms of neuronal apoptosis under normal physiological conditions and then will consider the role that apoptotic cell death plays in degenerative diseases. Finally, some examples of the role of apoptosis in mediating the effects of neurotoxicants will be considered.

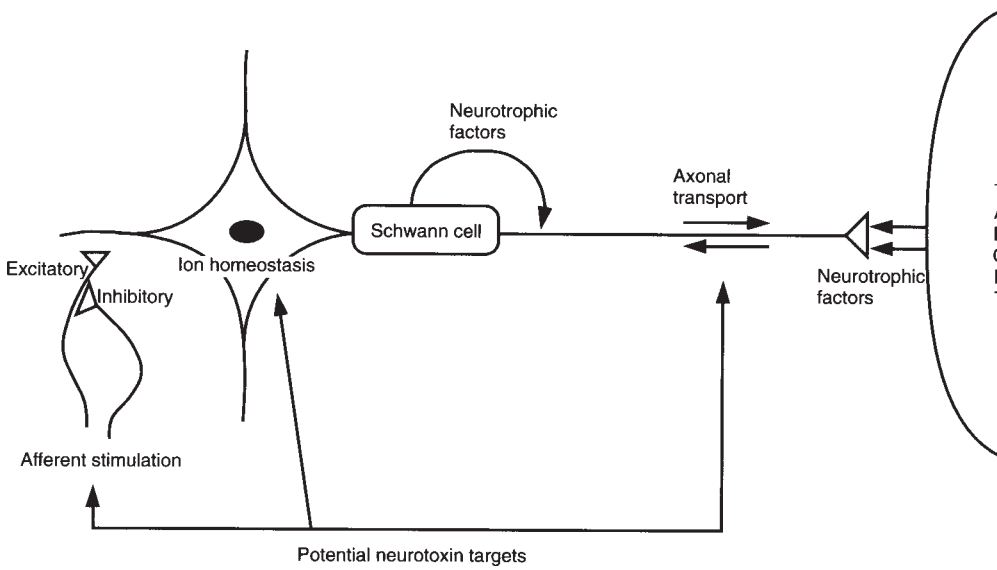

Figure 7.1 Neurons depend on multiple signals to survive.

7.2 NEURONAL APOPTOSIS

7.2.1 Detection and quantitation of neuronal apoptosis

Techniques are well developed for detecting apoptosis in proliferating non-neuronal cells, using for examples, chromatin stains, viability assays, detection of cleavage of cellular proteins by caspases and DNA fragmentation (reviewed in Chapter 10). Neuronal cells *in vitro* can be similarly assessed. Methods and reagents for looking for caspase activation in tissue samples and sections are being developed; it is possible to homogenise brain tissue, for example, and assay for DNA fragmentation and active caspases (Cheng *et al.,* 1998), but the TUNEL (or terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling) technique, which detects DNA strand breaks, remains one of the most widely used methods for detecting apoptosis *in situ* (Ben-Sasson *et al.,* 1995). Since DNA strand breaks are not confined to apoptosis, caution is needed when interpreting the results (Charriaut-Marlangue and Ben-Ari, 1995). However, the distinctions between neuronal apoptosis and necrosis may be blurred; neuronal cell death *in vivo* may not be so readily compartmentalized into either type (for discussion, see Baringa, 1998b).

7.2.2 Neuronal apoptosis during development

Neurones are over-produced during development and the excess are subsequently eliminated by apoptosis (reviewed in Oppenheim, 1991). It was generally thought that the deletion reflects competition for contact with targets and target-derived neurotrophic factors, so that neurones whose processes fail to reach their target die by apoptosis. In this way, the number of neurones is carefully matched with the targets which require innervation. Exogenous application of neurotrophic factors can prevent this neuronal death (Hofer and Barde, 1988) and antibodies to nerve growth factor (NGF) can induce neuronal death (Levi-Montalcini and Booker, 1960). In the sympathetic nervous system, for example, survival of neuronal precursor cells is initially independent of neurotrophic factors, allowing the cells to grow and proliferate. As the cells differentiate, they become increasingly dependent on NGF and neurones that fail to reach their target-derived supply of NGF die by apoptosis; in the rat, this begins at approximately day 19.5 (Birren *et al.,* 1993). Once connections are established, the extent of cell death declines and the neurones become less acutely dependent on neurotrophic factors. In fact, many neurones require trophic support from several sources, such as both their targets and the Schwann cells myelinating their processes (Snider, 1994). Furthermore, it is now clear that neurotrophic factors constitute just one of a complex array of signals upon which neurones depend for survival (Figure 7.1). They also depend upon afferent

stimulation, blockade or removal of which can induce cell death (Okado and Oppenheim, 1984, Galli-Resta *et al.,* 1993). These mechanisms lead to careful regulation of target innervation and stabilization of the adult neuronal networks.

7.3 GENETIC REGULATION OF NEURONAL APOPTOSIS

The molecular mechanisms underlying the response of cells to such survival signals involves the Bcl-2 family of proteins. The Bcl-2 protein itself, as would be predicted on the basis of data from non-neuronal systems (Chapter 1; Adams and Cory, 1998), can protect neuronal cells from cell death induced by a diverse array of stimuli (reviewed in Merry and Korsmeyer, 1997). Overexpression of Bcl-2 in neuronal cells *in vitro* can inhibit apoptosis: it can protect neuronally differentiated PC 12 cells and primary cultures of sympathetic neurones from cell death induced by NGF withdrawal (Garcia *et al.,* 1992, Zhong *et al.,* 1993). Some neuronal cells however are not protected by overexpression of Bcl-2 protein. For example, ciliary ganglion neurones overexpressing Bcl-2 die by apoptosis when ciliary neurotrophic factor is withdrawn (Allsopp *et al.,* 1993). *In vivo,* overexpression of Bcl-2 leads to decreased cell death in response to diverse stimuli including anti-tumour drugs or focal ischaemia induced by middle cerebral artery occlusion (Martinou *et al.,* 1994, Lawrence *et al.,* 1996). Likewise, transgenic mice expressing Bcl-2 targeted to the peripheral and central nervous systems by the neurone-specific enolase promoter, have more neurones than their wild-type counterparts and fewer cells die following axotomy (Farlie *et al.,* 1995). However, loss of function analyzes suggest that another member of the Bcl-2 family, Bcl-XL, may be more important than Bcl-2 in neuronal survival. Mice lacking Bcl-XL die at embryonic day 13, displaying extensive death of neurones throughout the developing brain and spinal cord (Motoyama *et al.,* 1995), whereas mice lacking Bcl-2 can live to one month of age albeit with progressive postnatal loss of sensory and motoneurones (Michaelidis *et al.,* 1996).

The Bcl-2 family also contains pro-apoptotic members, and Bax has been shown to play a role in neuronal cell death. Overexpression of Bax leads to neuronal cell death (Vekrellis *et al.,* 1997) and expression of both Bax and Bcl-2 (but not Bcl-XL) is developmentally regulated such that their levels decrease in the cortex and cerebellum during postnatal rat brain development (Vekrellis *et al.,* 1997). Mice lacking Bax have more facial motoneurones and more neurones in the superior cervical ganglia at birth than their wild-type siblings and the neurones show increased resistance to apoptosis in response to insults such as axotomy (Knudson *et al.,* 1995, Deckwerth *et al.,* 1996). In addition to these roles of the Bcl-2 family in modulating death responses, Bcl-2 plays a role in axonal growth and neuronal differentiation. When overexpressed in several neuronal cell lines, Bcl-2 enhances neurite outgrowth (Zhang *et al.,* 1996) and neurones isolated from mice lacking Bcl-2 show slower neurite outgrowth and maturation than neurones from wild-type mice (Hilton *et al.,* 1997, Middleton *et al.,* 1998).

TABLE 7.1
Characteristics of mice lacking caspases

Caspase deficiency	Life span of mice	Defects of -/- neurones	References
Caspase-1	Normal	None reported	Kuida *et al.*, 1995, Li *et al.*, 1995
Caspase-2	Normal	Facial motoneurone apoptosis during development accelerated, but final number of neurones equals w.t. Isolated sympathetic neurones died after NGF withdrawal faster than w.t. neurones	Bergeron *et al.*, 1998
Caspase-3	Maximum age 1–3 weeks	Reduced developmental neuronal apoptosis; increased numbers of neurones (and glia) giving ectopic masses between cerebral cortex, hippocampus and striatum	Kuida *et al.*, 1996
Caspase-9	Die before day 3	Reduced developmental neuronal apoptosis; expansion of ventricular zone and cortex	Hakem *et al.*, 1998, Kuida *et al.*, 1998
Caspase-11	Normal	None reported	Wang *et al.*, 1998

The growing family of enzymes called the caspases are responsible for cleaving key components of cells so that cellular processes are disabled and the cell is packaged for phagocytosis (reviewed in Cryns and Yuan, 1998, Thornberry and Lazebnik, 1998). Activation of caspases has been demonstrated during neuronal apoptosis and cell death can be prevented by caspase inhibitors such as *crmA* and z-Val-Ala-DL-Asp-fluoromethyl ketone (Gagliardini *et al.,* 1994, Deshmukh *et al.,* 1996) (see Chapter 2). Inhibition of caspases can protect sympathetic neurones from apoptosis induced by growth factor withdrawal (Deshmukh *et al.,* 1997) and can attenuate neuronal cell death *in vivo,* induced, for example, by ischaemic injury (Loddick *et al.,* 1996, Cheng *et al.,* 1998). This will be discussed in more detail in section 7.5.1. Strong evidence for a role for caspases in neuronal cell death comes from gene knockout mice (summarized in Table 7.1). Although mice lacking caspase-1 show no detectable neuronal deficits, neurones isolated either from these mice or from transgenic mice expressing a mutant form of caspase-1 are resistant to trophic factor withdrawal-induced apoptosis and the mice show reduced brain injury after ischaemic damage (Li *et al.,* 1995, Friedlander *et al.,* 1997). Interestingly, mice lacking caspase-2 display accelerated neuronal apoptosis suggesting that this enzyme has some neuroprotective properties. Furthermore, caspase inhibitors cannot block all neuronal apoptosis: apoptosis of cerebellar granule cells in response to potassium deprivation is unaffected by such inhibitors (Miller *et al.,* 1997, Taylor *et al.,* 1997). This may indicate the existence of a caspase-independent pathway to cell death. Alternatively, it may be that in some cells the decision to die (or commitment point) is made upstream of the caspases so that inhibiting the enzymes does not promote

long-term survival. These concepts of commitment and execution have been reviewed in Chapter 1.

One potent method to induce apoptosis in many cells is via activation of the tumour necrosis factor (TNF)/Fas receptors. These receptors contain death domains in their cytoplasmic tails. Upon binding ligand, the receptors trimerize and form a death inducing signalling complex (DISC) that directly leads to activation of caspase-8 (reviewed in Nagata, 1997). Another member of this receptor superfamily is the low-affinity neurotrophin receptor p75, which together with the Trk receptors Trk A, B and C, mediates cellular responses to neurotrophins. The mode of action of p75 is rather unusual in that, in contrast with the TNF receptor and Fas, it initiates a death signal when no ligand is bound. Thus when NGF is withdrawn from NGF-dependent cells, the cells die by apoptosis. Down-regulation of p75 expression can lead to resistance to apoptosis in response to NGF withdrawal. However, the existence of multiple neurotrophin receptors complicates this picture. In fact, the response to neurotrophic factors critically depends on which receptors are expressed and in what ratio (reviewed in Bredesen and Rabizadeh, 1997). When p75 and a Trk receptor are co-expressed and can both bind the neurotrophin present, co-operation between the two receptors generates binding sites of higher affinity than that of the Trk receptor alone. However, conversely, mutual repression can occur when the neurotrophin can bind only p75 and not the Trk receptor expressed on the cells, the signalling from one receptor then inhibits that from the other receptor. For example, the neuronal cell line PC12 expresses both p75 and Trk A. When serum is withdrawn, the cells die by apoptosis and whilst NGF can rescue the cells, brain-derived neurotrophic factor (BDNF) cannot. If the expression of Trk A is decreased, however, BDNF becomes anti-apoptotic (Taglialatela *et al.,* 1996). Similarly, introduction of Trk A into an oligodendrocyte line can suppress activation of the apoptotic signal from p75 (Yoon *et al.,* 1998). Thus these receptors appear to initiate complex interacting and merging signals that modulate the cellular response to neurotrophins.

7.4 WHAT MAKES NEURONES SUSCEPTIBLE TO TOXICANTS

Certain features of neurones make them susceptible to particular types of toxic insult either during the onset of disease or following administration of a toxicant. The first feature is their postmitotic phenotype and longevity. Because neurones are formed largely when mammals are born and retain very limited regenerative capacity into adulthood, they are susceptible to agents which can gradually accumulate to toxic levels.

The second feature is their structure. Neurones are typically 10,000 times longer than other cell types and have 1000 times more cell volume, most of which is in the axon. As a result, distal parts of the cell can be up to one metre from the cell body, yet protein synthesis is restricted to the cell body and some dendrites. Transport of proteins, vesicles and organelles is therefore essential for neuronal viability and axonal integrity. Axonal

transport depends on the cytoskeleton and can be broadly classified into slow and rapid transport. Rapid transport uses the molecular motors, kinesin and dynein, which require ATP. There are emerging connections between defects in axonal transport and some neurodegenerative disorders, suggesting that agents which interfere with transport can with time lead to neuronal apoptosis (see section 7.6).

The third cellular feature unique to neurones is the synapse. Functioning of the nervous system depends on information transfer within and between cells. Information transfer between cells occurs via chemical and electrical synapses and since neurones have been shown to depend on stimulation for survival, it follows that toxicants which interfere with synaptic function can ultimately lead to cell death. Conversely, over-stimulation of glutamate receptors can also lead to cell death (reviewed in Choi, 1992) although the mode of death remains controversial. The morphology of neurones dying by glutamate excitotoxicity is consistent with a necrotic cell death: the cell bodies and dendrites swell and organelles disintegrate. This leads in turn to further release of glutamate and propagation of the toxic signal, leading to the development of so-called secondary lesions.

7.5 NEURONAL APOPTOSIS AND DISORDERS OF THE CENTRAL NERVOUS SYSTEM

Despite controversies on the mode of neuronal cell death (see 7.2.1), it is generally accepted that neurons die by apoptosis during development. In contrast, the view that acute injury and neurodegeneration lead to necrosis has predominated. However, this has been challenged by observations of apparent apoptosis in these types of pathological neuronal death. Furthermore, it appears that genes implicated in neurodegeneration may actually participate in fundamental aspects of apoptosis, possibly even in developmental cell deaths. This is leading to exciting possibilities for the development of new intervention therapies for the treatment of a wider variety of neurological diseases, described below.

7.5.1 Ischaemic brain injury

Focal cerebral ischaemia can result from stroke, haemorrhage, cardiac or respiratory failure. It leads to neuronal injury, activation of glia and extravasation of leukocytes from brain microvessels. Although ischaemia was thought to induce necrosis rather than apoptosis on the basis of the morphology of the dying neurones, some experimental evidence now supports a role for apoptosis in ischaemic injuries (see Baringa, 1998b). For example, DNA fragmentation, a hallmark feature of apoptosis, has been observed in cells isolated from the ischaemic cortex (Linnik *et al.,* 1993). One neurotoxic stimulus during ischaemia

is thought to be the accumulation of high local concentrations of excitatory amino acids, such as glutamate. Following ischaemia, cytokines of the interleukin-1 family are induced, triggering an inflammatory response. IL-1ß is quickly expressed upon injury and although itself is not neurotoxic, it acts to mediate and exacerbate the injury caused by ischaemia (Loddick *et al.,* 1996). IL-1β is synthesized in a pro-form; activation requires the enzyme interleukin-1ß converting enzyme (ICE) or caspase-1. Thus, agents which inhibit production of IL-1ß can decrease the extent of damage resulting from ischaemia; administration of inhibitors of caspase-1 can reduce the damage in response to ischaemia (Loddick *et al.,* 1996). Transgenic mice expressing a mutated form of caspase-1, which is incapable of cleaving pro-IL-1ß, show significantly reduced injury in terms of both infarct volume and neurological score of the recovering mice compared to wild-type controls (Friedlander *et al.,* 1997). Mice deficient in caspase-1 also show reduced brain injury after middle cerebral artery occlusion (Schielke *et al.,* 1998). It is as yet unclear, however, what are the relative contributions of caspase-mediated production of proinflammatory IL-1ß and caspase-induced apoptosis (Baringa, 1998b). Caspases other than caspase-1 have also been implicated in ischaemic neuronal injury. Ischaemia induces activation of caspase-3: the active p20 subunit can be detected in neuronal cells at the time of reperfusion (Namura *et al.,* 1998), and expression of the messenger RNA and protein is up-regulated several hours later (Chen *et al.,* 1998). Caspase inhibitors can reduce the infarct size, brain swelling and neurological deficits (Hara *et al.,* 1997), even when administration is delayed until after the ischaemic insult (Cheng *et al.,* 1998), and caspase inhibitors given in conjunction with an excitatory amino acid antagonist can act synergistically to give greater neuroprotection after ischaemia (Ma *et al.,* 1998). Caspase-11 is required for activation of caspase-1 and mice with mutant caspase-11 are more resistant to ischaemic damage than wild-type mice (Wang *et al.,* 1998). In addition, overexpression of the anti-apoptotic proteins Bcl-2 or Bcl-XL can reduce the amount of damage following cerebral ischaemia (Martinou et al., 1994, Parsadanian *et al.,* 1998).

These results suggest that IL-1ß, caspases and excitatory amino acids all contribute to neuronal damage in response to ischaemia and that successful therapeutic intervention may be possible using a multiple hit strategy.

7.5.2 Chronic neurodegenerative diseases

Extensive neuronal death is a hallmark of neurodegenerative diseases as well as acute injury. Although most cases are sporadic, studies of families with hereditary forms of these diseases have yielded insight into some of the genes that potentially regulate the disorders and have given clues as to the nature of the neurotoxic stimulus as well as avenues to explore for therapies. Protein aggregation is a common feature of chronic neurological disorders. The mutated proteins form abnormal aggregates (Figure 7.2); the extent to which these contribute to the aetiology of the disorders is not completely clear.

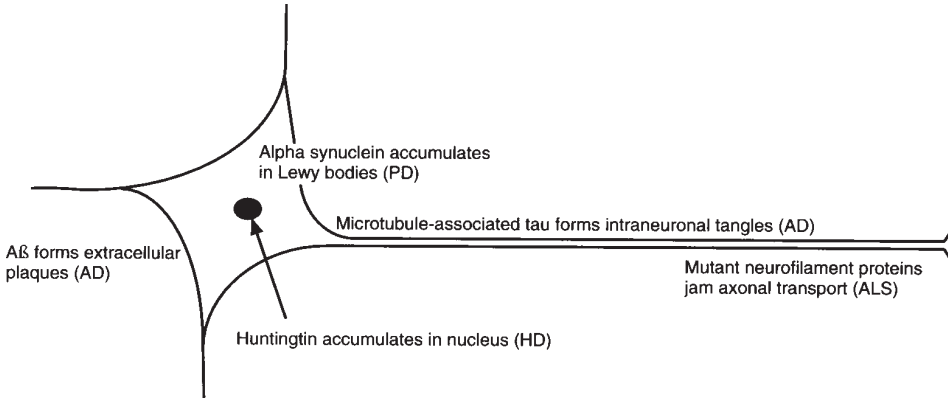

Figure 7.2 Protein aggregation is a common feature of chronic neurological disorders.

7.5.2.1 Alzheimer's disease

Alzheimer's disease (AD) leads to debilitating memory and cognitive losses. Patients develop extracellular deposits of a 40–42 amino acid amyloid-ß peptide (Aß) and intraneuronal tangles of highly phosphorylated tau, a microtubule associated protein. The Aβ peptides are produced from ß-amyloid precursor protein (ßAPP) by specific proteolytic cleavages and accumulate in deposits and tangles in areas of the brain which function in memory and cognition. The Aß deposits take several forms: Aß filaments, predominantly consisting of the abundant Aß(1–40) peptide, and non-filamentous deposits or diffuse plaques of Aß(1–42).

Three other genes have been found to be mutated in AD. Presenilins 1 and 2 are homologous proteins with multiple transmembrane segments. Mutations in presenilins may be neurotoxic in several ways: they may disrupt calcium homeostasis, lead to increased levels of neurotoxic Aß and/or increase caspase-mediated fragmentation of the proteins themselves. These mechanisms may then make cells more vulnerable to death either by the loss of a neuroprotective presenilin protein or generation of neurotoxic presenilin fragments.

The third gene found to be mutated in familial AD is apolipoprotein E (apoE). ApoE4 alleles lead to elevation of the density of the Aß deposits by an unknown mechanism. Some light may be shed on the mechanism however by the discovery of another protein implicated in AD pathology: a yeast two-hybrid screen for proteins that interact with Aß isolated a novel protein called endoplasmic-reticulum-associated binding protein (ERAB) (Yan *et al.,* 1997). ERAB is found predominantly in AD near Aß plaques but not in normal brain; overexpression of ERAB increases Aß toxicity whilst blocking ERAB decreases it. It may therefore be that it is the ERAB-Aß complex which is neurotoxic in AD. Interestingly, the protein

shares homology with the short chain alcohol dehydrogenases, which are involved in cholesterol biosynthesis, suggesting a connection between ApoE, cholesterol and AD.

Inflammation also plays a role in AD and anti-inflammatory drugs can delay the onset or reduce the rate of progression of the disease (Rogers *et al.,* 1996). Elevation of proinflammatory cytokines such as IL-1β has been observed (Patterson, 1995), and activated microglia, which can produce cytokines and other toxic factors such as free radicals, cluster near the Aβ plaques. One secreted form of βAPP can indeed induce such an inflammatory response in microglia. This can be blocked by ApoE3 but not the mutant ApoE4 alleles associated with AD, providing another potential link between this ApoE4 allele and the disease (Barger and Harmon, 1997).

7.5.2.2 Motoneurone disorders

Amyotrophic lateral sclerosis (ALS), or Lou Gehrig's disease, is an age-dependent neurodegenerative disease, leading to progressive paralysis and death typically within 3 to 5 years of diagnosis. The chief pathological feature is the degeneration of motoneurones in the brain, brainstem and spinal cord. The gene encoding the cytosolic copper-zinc superoxide dismutase (SOD1) was found to be mutated in several cases of familial ALS. The function of SOD is believed to be the detoxification of superoxide anions to form hydrogen peroxide which is subsequently converted to water by catalase or glutathione peroxidase. Mutation of SOD in ALS could thus increase reactive oxygen species to neurotoxic levels.

The mutations of SOD associated with ALS do not map to the active site of the enzyme (Deng *et al.,* 1993), however, but decrease its activity by decreasing the protein's stability more than its enzyme activity *per se* (Borchelt *et al.,* 1994). This suggests that the mutant SOD1 proteins are neurotoxic via a mechanism independent of the wild-type function of the enzyme. It is also possible that the altered enzymes have new substrate affinities which increase production of toxic reaction products.

The mutant forms of SOD generated may themselves be neurotoxic since transgenic mice overexpressing mutant SOD1 develop a neurodegenerative disorder closely resembling ALS, whilst mice overexpressing the wild-type form of the enzyme appear normal (Gurney *et al.,* 1994). Furthermore, it has been shown that whilst wild-type SOD1 inhibits apoptosis, mutated forms of SOD1, which retain enzymatic activity, induce apoptosis of neuronal cells in culture (Rabizadeh *et al.,* 1995).

The neuronal apoptosis which occurs in response to SOD1 down-regulation can be inhibited by peptide inhibitors of caspase-1, as well as by IL-1ra and blocking antibodies to IL-1β (Troy *et al.,* 1997). Further evidence for a role of apoptosis in ALS comes from the demonstration that overexpressing the neuroprotective Bcl-2 protein in SOD1 mutant mice delays the onset of the disease and prolongs survival (Kostic *et al.,* 1997).

Impaired axonal transport is also a feature of ALS, and this may partly explain the acute susceptibility of the long motoneurones. Neuronal aggregates of neurofilament

proteins occur in ALS patients and these could impair the transport critical for distal neuronal viability. Mutation of neurofilament proteins have been reported in this disease (Figlewicz *et al.,* 1994) and mice overexpressing either wild-type or mutant neurofilament proteins develop motoneurone disorders (Cote *et al.,* 1993). Furthermore, transgenic mice expressing SOD1 mutants develop defects in axonal transport before any other obvious pathology (Williamson *et al.,* 1997).

Another motoneurone disorder is spinal muscular atrophy (SMA), which is classified into types I to III according to the severity of the disease and age of onset, with type I patients dying within their first two years, whilst type III patients do not present with symptoms until this age. SMA is characterized by loss of motoneurones and accompanied by progressive weakness and wasting of muscles and finally paralysis. Two genes have been linked to the disease: evidence suggests that mutation of either gene could be neurotoxic and lead to increased apoptosis and motoneurone loss. The gene product of Smn, or survival motoneurone (Lefebvre *et al.,* 1995), has been proposed to be involved in maturation of nuclear RNA (Liu and Dreyfuss, 1996) but, until quite recently, the mechanism whereby Smn affects motoneurone survival was unclear. Smn was then reported to interact with Bcl-2 and enhance its anti-apoptotic effect, although Smn alone is not antiapoptotic (Iwahashi *et al.,* 1997). In contrast, the identity of the second gene implicated in SMA immediately suggested how mutation can lead to aberrant apoptosis: neuronal apoptosis inhibitor protein (NAIP) (Roy *et al.,* 1995) was the first mammalian homologue of the viral IAP or inhibitor of apoptosis proteins to be described (reviewed in Clem and Duckett, 1997). NAIP is related to the viral IAPs since it shares a similar protein domain structure and it has been shown to have anti-apoptotic activity in mammalian cells (Liston *et al.,* 1996). Furthermore, it is located in motor but not sensory neurones which is consistent with the fact that motoneurones are selectively targeted in SMA.

7.5.2.3 Huntington's disease

Huntington's disease (HD) is an autosomal dominant genetic disorder, characterized by progressive movement disturbances and devastating cognitive and psychiatric deterioration, leading to death within 10 to 15 years of disease onset. The major targets are the neurones of the basal ganglia and striatum, especially the γ-aminobutyric acid producing medium spiny neurones.

The genetic alteration associated with the disorder is expansion of an unstable trinucleotide (CAG) repeat within the HD gene (Huntington's Disease Collaborative Research Group, 1993). The size of the CAG repeat varies from 8 to 35 in normal individuals and from 40 to 70 or more in affected individuals and the longer the repeated region, the earlier the onset of the disease. The function of the normal huntingtin protein and the basis for the neurotoxicity of the mutant forms with their elongated stretches of glutamines are unclear. Like SOD1, huntingtin is widely expressed in the body, leaving

us to wonder at the basis for the selective neurotoxicity of the disease. It may be that proteins which interact with huntingtin or SOD1 lead to specific targeting. In the brain, huntingtin is present in the cytoplasm and associated with vesicle membranes in neurones, suggesting a potential role in vesicle trafficking (Difiglia *et al.,* 1995).

For all these neurodegenerative disorders, animal models which faithfully reproduce the human features of the disease are invaluable for studying mechanisms of neurotoxicity as well as for evaluating potential therapies. Disruption of the HD gene in mice is lethal beyond embryonic day 8.5 demonstrating that huntingtin is essential for post-implantation development. The heterozygous mice have a phenotype resembling human HD to some extent, including loss of neurones in the basal ganglia and loss of cognitive flexibility (Nasir *et al.,* 1995). These results suggest that there is no functional redundancy for the HD gene. Since humans with expanded CAG repeats in the HD gene survive, this also suggests that the poly-glutamine region does not obliterate the wild-type function of the protein, but the neurotoxicity may rather reflect a novel, selectively lethal gain of function.

Huntingtin is cleaved by caspase-3 and the rate of cleavage increases with expansion of the poly-glutamine region (Goldberg *et al.,* 1996). Since mice lacking huntingtin show higher than normal levels of apoptosis in the embryonic ectoderm (Zeitlin *et al.,* 1996), it may be that the story resembles that of the presenilins in AD: wild-type huntingtin may be anti-apoptotic, but following poly-glutamine expansion, the protein may become more susceptible to caspase-mediated cleavage, the fragments aggregating and becoming neurotoxic.

7.5.2.4 Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative disorder characterized by death of the dopaminergic neurones of the substantia nigra. Onset of the disease is rare before the age of 40 and is accompanied by symptoms which include resting tremor, muscular rigidity, postural instability and bradykinesia. These motor symptoms result from an imbalance of inputs into the striatal neurones; loss of the neurones of the substantia nigra denies the striatal neurones of inhibitory input and they therefore respond with increased activity to excitatory cortical inputs. As with the other neurodegenerative disorders discussed, the exact nature of the neurotoxic stimulus in PD remains unresolved. Experimental evidence suggests a role for oxidative stress in many of the diseases (reviewed in Simonian and Coyle, 1996).

Some cases of PD are associated with mutation in a gene for α-synuclein (Polymeropoulos *et al.,* 1997). Alpha synuclein is a protein of unknown function found in presynaptic nerve termini. It has been found in Lewy bodies of the substantia nigra in patients with PD (Spillantini *et al.,* 1997); Lewy bodies are intracellular inclusion bodies characteristic of but not unique to PD. They also contain neurofilaments, ubiquitin and other components of the protein degradation pathway (Mezey *et al.,* 1998). Mutation of α-synuclein may facilitate its aggregation and play a role in formation of Lewy bodies, but how these events

lead to neurotoxicity of dopaminergic neurones is unclear; the aggregates may be neurotoxic and/or disrupt the normal function of the protein. However it is important to note that α-synuclein is probably only a minor locus for familial forms of PD (French Parkinson's Disease Genetics Study Group, 1998). Indeed, α-synuclein-containing inclusions are found in other neurodegenerative disorders (Mezey *et al.,* 1998, Clayton and George, 1998) whether they are causative or occur in response to neuronal damage is as yet unclear.

Connections between a-synuclein and neuronal cell death are so far lacking. However, experiments have shown that the neurotoxins MPP+ and 6-hydroxydopamine, which also generates PD-like symptoms in animals, can induce apoptosis in neuronal types of cells *in vitro* (Dipasquale *et al.,* 1991, Walkinshaw and Waters, 1994). Furthermore, striatal neurones undergo apoptosis in animal models of PD and the cell death is blocked by glutamate antagonists suggesting these cells die in response to the excitotoxic stimulus (Mitchell et al., 1994).

7.6 APOPTOSIS IN NEUROTOXICOLOGY

As discussed previously, neurones are particularly susceptible to cell death but the response of neuronal cells to neurotoxic stimuli is complex, reflecting the environment of the cells and the ability of the array of proteins expressed to respond to these environmental signals. For example, *in vivo,* overexpression of Bcl-2 leads to decreased cell death in response to diverse stimuli including the anti-tumour drug adriamycin or focal ischaemia induced by middle cerebral artery occlusion (Martinou *et al.,* 1994, Lawrence *et al.,* 1996). Similarly, most caspases can act as potent neurotoxins whereas inhibition of caspases can protect sympathetic neurones from apoptosis induced by growth factor withdrawal; these cells can recover fully their response to nicotine, when the growth factor levels are restored (Deshmukh *et al.,* 1997). Furthermore, there is evidence to suggest that inhibiting caspases can attenuate neuronal cell death *in vivo,* induced, for example, by ischaemic injury (Loddick *et al.,* 1996, Cheng *et al.,* 1998).

7.6.1 Glutamate

Information transfer between cells occurs via chemical and electrical synapses and since neurones have been shown to depend on stimulation for survival, it follows that toxicants which interfere with synaptic function can ultimately lead to cell death. Conversely, overstimulation can also lead to cell death. Thus, the accumulation of high local concentrations of excitatory amino acids can serve as a neurotoxic stimulus (reviewed in Martin *et al.,* 1998). Glutamate can function as a potent excitotoxin for neuronal cells and glutamate-induced excitotoxicity has been linked to both acute and chronic neurological disorders such as ischaemia or HD (reviewed in Martin *et al.,* 1998). The over-stimulation of glutamate receptors leads to an excessive rise in

intracellular calcium which contributes to the neurotoxicity (reviewed in Choi, 1992). The mode of cell death remains controversial. The morphology of neurones dying by glutamate excitotoxicity is consistent with a necrotic cell death: the cell bodies and dendrites swell and organelles disintegrate. This leads in turn to further release of glutamate and propagation of the toxic signal and development of so-called secondary lesions.

Evidence suggests that when cerebellar granule cells are treated with glutamate, two phases of cell death are observed; first, there is a rapid phase of necrosis but, subsequently, those neurones which have survived this phase and recovered their mitochondrial membrane potential and energy levels, then go on to die by apoptosis (Ankarcrona *et al.,* 1995). A role for apoptosis in glutamate-induced cell death is also suggested by the observation that it is accompanied by activation of caspase-3 and can be blocked by caspase inhibitors (Du *et al.,* 1997, Tenneti *et al.,* 1998).

7.6.2 Other excitatory amino acids

As discussed above, toxicants which interfere with synaptic function can ultimately lead to cell death (reviewed in Martin *et al.,* 1998). Interestingly, the pathology associated with HD can be reproduced in animal models by intrastriatal injection of the glutamate receptor agonist kainic acid (McGeer and McGeer, 1976) or injection of the NMDA receptor agonist quinolinic acid. The latter is reported to give rise to a pathology which more closely resembles that of HD (Beal *et al.,* 1986). The contribution of apoptosis to the neuronal cell death seen in HD is not completely clear and may, like other examples of excitotoxic death, have both necrotic and apoptotic components.

7.6.3 Aß

Aß is a peptide deposited in the brain of patients suffering from Alzheimer's disease, specifically in regions which function in memory and cognition. Aß has been shown to be neurotoxic since aggregates of the peptide induce primary cultures of neuronal cells to become dystrophic and to die by apoptosis (Loo *et al.,* 1993). Similarly, primates display neuronal degeneration in response to intracerebral injection of the Aß peptides (Geula *et al.,* 1998). This is both species- and most importantly age-dependent (Geula *et al.,* 1998). Curiously, transgenic mice overexpressing the precursor protein develop Aß aggregates, but do not show the neuronal death associated with human AD (Quon *et al.,* 1991). This may be explained since the toxicity of Aß is significantly greater in the aged brain (Geula *et al.,* 1998). The molecular mechanisms by which Aß induces neuronal apoptosis remain elusive (Baringa, 1998a). However, sublethal doses of Aß can sensitize neurones to other challenges such as exposure to excitotoxic amino acids or glucose deprivation.

7.6.4 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)

1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine or MPTP is a piperidine derivative that causes irreversible symptoms of parkinsonism in humans and monkeys. It has been identified as an impurity in isomeperidine, a designer drug also known as synthetic heroin since people using isomeperidine developed PD-like symptoms. In glial cells, MPTP is converted by monoamine oxidase B into the neurotoxin 1-methyl-4-phenylpyridinium (MPP+) and MPP+ is subsequently taken up into the nerve termini of dopaminergic neurones via their dopamine transporter, explaining the selective targeting to dopaminergic neurones. Once inside the cells, MPP+ inhibits the mitochondrial electron transport chain, leading to cell death. The clinical effects of MPTP closely resemble the symptoms of PD and the drug can be used to generate animal models of the disease (Burns *et al.,* 1983). Inhibition of electron transport has been reported in idiopathic PD (Schapira *et al.,* 1990) and the effects of MPTP appear age-related explaining the late onset of PD (reviewed in Langston, 1996).

7.6.5 Toxicants that target axonal transport

Axonal transport is dependent upon a functional cytoskeleton and upon the activity of molecular motors. These molecular motors, kinesin and dynein are dependent upon ATP and drive axonal transport along cytoskeletal microtubules. Thus, axonal transport is susceptible to toxicants that are metabolic inhibitors and prevent the formation of ATP such as cyanide. Similarly, toxicants that effect microtubule function such as the chemotherapeutic drug taxol are able to inhibit the function of the molecular motors. There are emerging connections between defects in axonal transport and some neurodegenerative disorders, suggesting that agents which interfere with transport can, with time, lead to neuronal apoptosis.

7.7 SUMMARY

Understanding neurotoxic stimuli and the cellular responses underlying neuronal cell death is critical if we are to protect humans from potentially harmful toxicants whilst developing effective therapies for such devastating disorders as Parkinson's and Alzheimer's. More knowledge is required on the aetiology of these diseases and on the functions of the proteins implicated. Although the distinction between apoptosis and necrosis may be blurred in CNS cells, the surge of interest in apoptosis and the mechanisms of cell death will hopefully help us answer at least some questions and develop effective strategies for intervention.

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CHAPTER 8

Perturbation of Apoptosis as a Mechanism of Action of Nongenotoxic Carcinogens

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8.1 Introduction
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8.1 INTRODUCTION

Nongenotoxic carcinogens are a group of structurally diverse compounds that cause cancer in rodents without damaging DNA (reviewed in Ashby *et al.,* 1994). A number of mechanisms have been proposed to explain their mode of action. Generation of reactive oxygen species, induction of DNA synthesis and suppression of apoptosis have been suggested as possible mechanisms for the carcinogenicity observed in rodent bioassays (Ashby *et al.,* 1994).

The liver is the major target organ for carcinogenicity (Ashby and Tennant, 1991) and there are several classes of nongenotoxic carcinogen that can cause hepatocellular adenomas and carcinomas. These include barbiturate drugs such as phenobarbitone (PB) (Butler, 1978), dioxins such as 2, 3, 7, 8-tetradichlrobenzo-b, e-1,4-dioxin (TCDD) (Poland and Knutson, 1982) and the largest group, the peroxisome proliferators (reviewed in Ashby *et al.,* 1994). Despite the prevalence of liver tumours, neoplasia is observed in other tissues. For example, tetradecanoylphorbol ester causes skin carcinoma (Balmain and Pragnell, 1983, Bremner *et al.,* 1994), trimethylpentane is the chemical implicated in the nephrocarcinogenicity of unleaded petrol (Lock, 1990) and monoethylhexylphthalate (MEHP), a metabolite of the plasticizer DEHP, can cause tumours in rat testes (Richburg and Boekelheide, 1996).

8.2 NONGENOTOXIC CARCINOGENS AND CANCER

The carcinogenic potential of different nongenotoxic carcinogens differs considerably. Peroxisome proliferators (PPs) are a group of chemically distinct compounds which include chemicals of therapeutic, industrial and environmental significance such as hypolipidaemic fibrate drugs, leukotriene antagonists, clingwrap/medical tubing, plasticizers, herbicides and de-greasing solvents (Moody *et al.,* 1991, Green, 1992, Ashby *et al.,* 1994) (Figure 8.1). Continued treatment of rodents with PPs results in the development of hepatic adenomas and carcinomas (Reddy *et al.,* 1980). The potent non-fibrate hypolipidaemic drug Wyeth-14,643 and the leukotriene antagonist LY-171883 cause 100% tumours at 2 years in rats (0.1% w/w) in the diet (Marsman *et al.,* 1988). Weaker chemicals, such as DEHP and trichloroethylene yield hepatocellular carcinomas at 2 years when administered at 1.2%–2.5% (w/w) and 1200 mg/kg, respectively (National Toxicology Program, 1983). Although proliferation of peroxisomes is observed upon administration of PPs, it has become apparent that peroxisomal proliferation alone cannot account for tumourigenesis. Weak hepatocarcinogens such as DEHP only cause 25% less peroxisome proliferation when compared with potent carcinogens such as Wyeth-14,643 (Moody *et al.,* 1991). In addition, trichloroacetic acid (TCA) stimulates peroxisome proliferation in both rats and mice but only induces DNA synthesis and is only carcinogenic in mouse (Herren-Freund *et al.,* 1987).

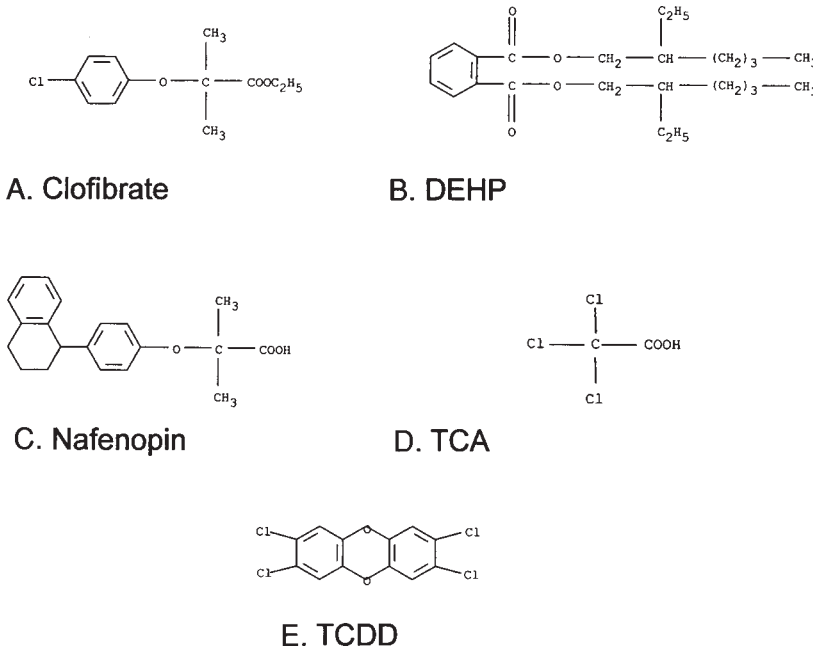

Figure 8.1 Structures of some members of the peroxisome proliferator class of rodent nongenotoxic hepatocarcinogens.

The nongenotoxic liver carcinogen phenobarbital has also been studied extensively (Schulte-Hermann *et al.,* 1990, Phillips *et al.,* 1997). At doses between 400 ppm and 700 ppm, this compound causes more than 30% liver enlargement (Berman *et al.,* 1983, Edwards and Lucas, 1985, Mansbach *et al.,* 1996) probably due to an induction of hepatic DNA synthesis (Berman *et al.,* 1983, Edwards and Lucas, 1985) coupled with suppression of apoptosis (Oberhammer and Qin, 1995, James and Roberts, 1996, Worner, 1996). There are numerous studies showing that PB is hepatocarcinogenic in the rat liver (Rossi *et al.,* 1977, Phillips *et al.,* 1997). However, some authors suggest that PB is not a complete carcinogen but merely acts as a promoter of genotoxic damage caused by compounds such as diethylnitrosamine (DEN) (Peraino *et al.,* 1975, Butler, 1978, Neveu *et al.,* 1990, Worner, 1996). Treatment with PB and other barbiturates causes a number of changes in the expression of different classes of enzymes, such as aldehyde dehydrogenases and the cytochrome p450 mono-oxygenases as well as changes in the levels of a number of peroxisomal, microsomal, cytosolic and mitochondrial enzymes in hepatocytes *in vitro* and *in vivo* (Baron *et al.,* 1981, Liang *et al.,* 1995).

The biochemical and molecular mechanisms by which nongenotoxic carcinogens such as PB or PPs cause liver cancer is still unclear. The data available to date suggest that changes in cell proliferation and apoptosis are likely to play an important role. However, other mechanisms cannot be ruled out and may act alongside hepatic growth perturbation.

8.3 SPECIES DIFFERENCES IN RESPONSE TO NONGENOTOXIC CARCINOGENS

There are marked species differences in the response to nongenotoxic carcinogens (Lake *et al.,* 1989, James and Roberts, 1995, Latruffe, 1997). In rat or mouse, hepatocarcinogens such as the PPs or PB cause liver enlargement, hepatocyte replication and induction of detoxification enzymes such as cytochrome p4504A1 and 2B1/2, respectively (Moody *et al.,* 1977, 1991, Berman *et al.,* 1983, Edwards and Lucas, 1985, Mansbach *et al.,* 1996, Bell *et al.,* 1998). However, experiments using cultured human hepatocytes and data from patients receiving fibrate drug therapy suggest that humans do not display the adverse effects of PPs observed in rats and mice (Elcombe and Styles, 1989, Perrone and Williams, 1998). Specifically, human hepatocytes *in vitro* show no peroxisome proliferation nor hepatocyte proliferation and there appears to be no increased risk of developing hepatoadenomas in patients receiving peroxisome proliferators such as fenofibrate and bezafibrate (Ashby *et al.,* 1994). However, humans do respond to the fibrate PPs by altered expression of enzymes that regulate serum cholesterol and lipid homeostasis (Devchand *et al.,* 1996, Schoonjans *et al.,* 1996b). These PP-mediated changes form the basis of the clinical benefit to patients at risk of ischaemic heart disease. Such marked species differences in the nature and magnitude of the response to nongenotoxic carcinogens suggest that, despite their obvious carcinogenic potential in rodents, nongenotoxic carcinogens such as the PPs pose no risk to human health (Cattley *et al.,* 1998).

8.4 MECHANISM OF ACTION OF NONGENOTOXIC CARCINOGENS

8.4.1 Transcriptional activation by nongenotoxic carcinogens

The biological effects of many nongenotoxic carcinogens appear to be receptor-mediated (Green, 1992), where the toxicant binds to an intracellular receptor and perturbs growth regulation via a direct or indirect effect on gene expression. For example, TPA mimics diacylglyerol (DAG) by binding to and stimulating protein kinase C to switch on gene expression in the skin via the transcription factor AP1 (Mills and Smart, 1989, Sassone-Corsi *et al.,* 1990). PPs activate the peroxisome proliferator activated receptor (PPARa) (Issemann and Green, 1990) which has been shown to be responsible for the pleiotropic effects of these compounds (Lee *et al.,* 1995, Gonzalez, 1997a, Peters *et al.,* 1997). PPARa is expressed highly in hepatocytes, cardiomyocytes, enterocytes and the proximal tubules of the kidney (Issemann and Green, 1990, Braissant *et al.,* 1996). In the liver, activation of PPARa mediates the transcription of a number of genes, including CYP4A1 which encodes the microsomal enzyme cytochrome P4504A1 (Aldridge *et al.,* 1995) and the peroxisomal enzymes of ß-oxidation such and acyl-CoA oxidase, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase (Tugwood *et al.,* 1992, Aldridge *et al.,* 1995) (Table 8.1). In addition,

TABLE 8.1
Examples of PPARa inducible genes

Gene	Function
Acyl-CoA oxidase	Fatty acid β-oxidation
Enoyl-CoA hydratase-3-hydrixyacyl-CoA dehydrogenase	Fatty acid β-oxidation
C-acyl-CoA synthetase	Conversion of fatty acids into acyl-CoA derivatives
CYP4A6	Formation of dicarboxylic acids by ω-oxidation
aP2 (adipocyte fatty acid binding protein)	Adipose tissue fatty acid metabolism
Lipoprotein lipase	Hydrolysis of triglyceride-rich particles
Apolipoprotein A-I	Protein component of High Density Lipoprotein
Apolipoprotein A-II	Protein component of High Density Lipoprotein

activation of PPARα modulates high density lipoprotein (HDL) cholesterol levels by increased transcription of the major HDL apolipoproteins, apoAI and apoAII, and reduces plasma triglycerides via induction of LPL and reduction of apoCIII (Auwerx, 1992, Schoonjans *et al.,* 1996a, Latruffe and Vamecq, 1997). The ability to up-regulate expression of the P450 family of genes is common to other nongenotoxic carcinogens such as dioxin and PB (Baron *et al.,* 1981, Bains *et al.,* 1985, Bell *et al.,* 1991, Liang *et al.,* 1995), probably because of the key role played by the cytochrome P450 gene family in oxidative metabolism and detoxification.

PPARα activation by its ligands is essential for PP-mediated transcription of known target genes (Table 8.1), as well as for the induction of cellular proliferation. Transcriptional regulation by PPARα is achieved through PPAR-RXR (where RXR is the receptor for *9-cis* retinoic acid) heterodimers which bind DNA motifs called PPAR response elements (PPREs) within the promoters of their target genes (Tugwood *et al.,* 1992). Studies using PPARa null mice have indicated a complete lack of transcriptional response to chronic treatment with PPs (Lee *et al.,* 1995). In addition, PPARα null mice do not exhibit the increase in DNA synthesis normally observed upon administration of PPs (Lee *et al.,* 1995, Gonzalez, 1997a). This indicates that the adverse effects of PPs such as liver growth and cancer are mediated by activation of PPARα and are likely to involve transcription of new genes (Lee *et al.,* 1995, Gonzalez, 1997b, Peters *et al.,* 1997).

8.4.2 Generation of oxidative damage by nongenotoxic carcinogens

PB and PPs have been suggested to exert their tumour promoting activities by increasing cellular oxidative damage (Reddy, 1989, Chu *et al.,* 1995). Active oxygen, in the form of superoxide or hydrogen peroxide can be produced by an increase in peroxisomal fatty acid ß-oxidation coupled with an increase in catalase levels (Nilakantan *et al.,* 1998).

The resultant oxidative stress can lead to a number of diverse effects within the cell, including DNA damage, lipid peroxidation and new gene expression, which may lead to tumourigenesis (Cerutti and Trump, 1991). Although an increase in hydroxyl radicals is observed in rats given PPs (Reddy, 1989), conflicting data exist on the generation of DNA damage as an indirect mode of PP action (Huber *et al.,* 1997, Soliman *et al.,* 1997). Active oxygen species can directly modulate transcription through antioxidant response elements (AREs) found in the promoters of certain genes (Pinkus *et al.,* 1993). For example, the aryl hydrocarbon (Ah) gene battery comprises a family of at least six genes that respond to oxidative cytotoxicity (Nebert *et al.,* 1993). Other nongenotoxic carcinogens such as PB can also activate the promoters for glutathione-S-transferase Ya subunit and NAD(P)H: oxidoreductase genes through AREs (Pinkus *et al.,* 1993). Active oxygen species, in turn, can induce transcription through members of the nuclear factor-κB (NF-κB) family of transcriptional regulators (Nilakantan *et al.,* 1998). Thus, oxygen radicals may play a role as secondary messengers for a variety of agents.

8.4.3 Perturbation of growth by nongenotoxic carcinogens

It has been proposed that the tumourigenic potential of nongenotoxic carcinogens arises from their ability to promote the survival and growth of spontaneously initiated hepatocytes (Schulte-Hermann, 1983, Cattley *et al.,* 1991). This hypothesis arises from the observation that PPs and PB can cause cellular proliferation in the liver (Berman *et al.,* 1983). The majority of hepatocarcinogenic PPs cause measurable induction of DNA synthesis during the first 2–7 days of administration (Ashby *et al.,* 1994) but, frequently, this is not sustained and may return to control levels by around 10–20 days. However, the highly carcinogenic PP Wyeth-14,643 causes both acute and sustained hepatocyte proliferation in rat livers (Marsman *et al.,* 1988) leading to suggestions that carcinogenesis arises from prolonged mitogenic stimulation. The relative contribution of acute versus sustained proliferation remains to be determined.

The tightly controlled balance exerted in normal tissues between cell growth and cell death is disrupted during chemical-induced carcinogenesis (Roberts *et al.,* 1997). Thus, as well as stimulating proliferation, nongenotoxic carcinogens may alter rates of cell death in their target organs. This has been demonstrated for the PPs class of nongenotoxic hepatocarcinogens and also for PB since these chemicals suppress apoptosis in rodent hepatocytes (Bursch *et al.,* 1984, Schulte-Hermann *et al.,* 1990, Bayly *et al.,* 1994, Gill *et al.,* 1998, Roberts *et al.,* 1995). Induction of apoptosis by a number of stimuli, including the physiological liver factor $TGFß_1$, the DNA damaging agent etoposide or activation of the Fas receptor, can be suppressed in cultured rodent hepatocytes by PPs (Gill *et al.,* 1998). In addition to a reduction in the number of cells exhibiting apoptotic morphology, incubation with PPs or with PB results in maintenance of viability over several weeks, in contrast to eight days in control cultures (Bayly *et al.,* 1994). *In vivo* studies using other

nongenotoxic carcinogens have shown that although liver hyperplasia is observed upon administration of compounds such as cyproterone acetate or phenobarbital, withdrawal of these compounds causes regression of the hyperplastic liver with a concomitant increase in apoptosis (Schulte-Hermann *et al.,* 1980, Bursch *et al.,* 1984, 1985, Roberts *et al.,* 1995). This liver regression is reversible and can be inhibited by re-administration of the relevant compound.

8.4.4 Molecular mechanism for the suppression of apoptosis by nongenotoxic carcinogens

8.4.4.1 Cytokine networks

The molecular mechanism of the perturbation of growth by nongenotoxic hepatocarcinogens is unknown, but recent evidence suggests the involvement of cytokine signalling between different cell populations within the liver. In particular, tumour necrosis factor a (TNF-α) (Beutler and Cerami, 1988) has been implicated in mediating the growth perturbation effects of nongenotoxic hepatocarcinogens (James, 1998, Roberts and Kimber, 1999). Although TNF-α is known to be an inflammatory mediator proximally associated with necrotic injury (Deviere *et al.,* 1990), it may also have a role as a positive mediator of hepatocyte growth (Taub, 1996, Roberts and Kimber, 1999). The observation that TNFRI null mice show reduced liver regeneration after partial hepatectomy (Yamada *et al.,* 1997) has lead to the hypothesis that TNF-α may be involved in mediating the effects of nongenotoxic carcinogens (Rolfe *et al.,* 1997). Further studies have shown that TNF-α could mimic the effects of PPs in rat hepatocytes and that antibodies to TNF-α and TNFRI could attenuate the effects of PPs on DNA synthesis (Bojes *et al.,* 1997) and apoptosis (Rolfe *et al.,* 1997, West *et al.,* 1999). Thus, low levels of TNF-α produced from the resident liver macrophages, the Kupffer cells, may be mediating the effects of PPs on liver homeostasis in a paracrine signalling mechanism (Rose *et al.,* 1997). This hypothesis is supported by the observation that Kupffer cells are required for stimulation of mitosis by PPs (Rose *et al.,* 1997) and that PPs stimulate Kupffer cells (Bojes and Thurman, 1996, Rusyn, 1998). The molecular mechanism of putative TNF-α production and/or release in response to PPs is unclear at present.

TNF-α signalling involves the activation of both pro- and anti-apoptotic processes (Heller and Kronke, 1994, Smith *et al.,* 1994, Wallach, 1997). The anti-apoptotic effects of TNF-α are thought to be mediated by activation of novel gene expression, perhaps through activation of NF-κB (DiDonato *et al.,* 1997, May and Ghosh, 1998, West *et al.,* 1999). NF-κB, a critical regulator of cytokine-inducible gene expression, is activated by TNF-α and by pro-inflammatory cytokines such as IL-1 (Heller and Kronke, 1994, DiDonato *et al., 1997,* Malinin *et al.,* 1997) (Figure 8.2). Normally, NF-κB is held in an inactive state in the cytoplasm by IκB inhibitory proteins (DiDonato *et al.,* 1997). When cells

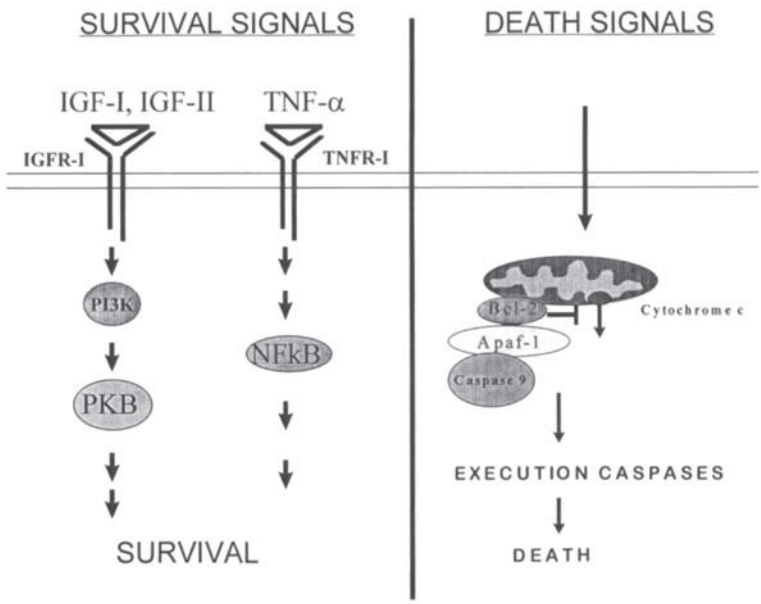

Figure 8.2 Cell survival and cell death signalling pathways.

are treated with TNF-α or IL-1, protein kinase cascades are activated that lead to the phosphorylation of IκB proteins. This targets IκB for ubiquitination and proteasome mediated degradation, allowing nuclear transport and activation of NF-κB (DiDonato *et al.,* 1997). NF-κB has been shown to be a pivotal transcription factor in liver regeneration (Cressman *et al.,* 1994, Beg *et al.,* 1995). NF-κB activity is induced during liver regeneration after partial hepatectomy, and inhibition of NF-κB by a dominant mutant of IκB results in the induction of massive hepatocyte apoptosis (Hellerbrand *et al.,* 1998, Iimuro *et al.,* 1998). To further support the role of NF-κB as an anti-apoptotic factor, disruption of the NF-κB p65 (RelA) subunit leads to embryonic lethality at 15–16 days of gestation, concomitant with extensive apoptosis of the liver (Beg *et al.,* 1995).

The NF-κB inducible cytokine IL-6 has also been shown to be essential for liver regeneration after partial hepatectomy, indicating that IL-6 might be a critical component of the regenerative response (Cressman *et al.,* 1996). Therefore, direct or indirect activation of cytokines such as TNF-α, IL-1 or IL-6 may be a common mechanism for the suppression of apoptosis by nongenotoxic carcinogens.

8.4.4.2 Activation of survival pathways

Hepatocyte survival is likely to be dependent on a number of factors. The regulation of cytokine networks may allow interactions with a number of other pathways which in

turn may cause suppression of apoptosis (Figure 8.2). Recent evidence suggests that the basic mechanisms for regulation of apoptosis are tied to cellular proliferation and transformation pathways (Harrington *et al.,* 1994). This co-ordination is illustrated by the observation that serum specific mitogens and growth factors inhibit apoptosis and promote survival (Harrington *et al.,* 1994). A number of survival factors have been described, including insulin-like growth factors I and II (IGF I and II) (Christofori *et al.,* 1994, Resnicoff *et al.,* 1995a) (Figure 8.2). IGFs are mitogenic polypeptides which are thought to be required in several cell types for the establishment and maintenance of transformed phenotypes and tumourigenesis (Resnicoff *et al.,* 1995b). IGFs are synthesized primarily in the liver and are thought to play an important role in the development of certain tumours (Daughaday and Rotwein, 1989). IGF-I mRNA levels have been shown to be high in the adult rat liver, whereas levels of IGF-II are low (Daughaday and Rotwein, 1989). However, hepatoma tissues frequently up-regulate expression of IGFs. In hepatocytes infected with virus, expression of IGF-II increases cell survival and promotes tumourigenesis (Ueda and Ganem, 1996). Furthermore, tumours develop with reduced malignancy and with a higher incidence of apoptosis in mice that are deficient in IGF-II (Christofori *et al.,* 1994). Thus, it is possible that nongenotoxic carcinogens may act by regulating IGF survival pathways in the liver either directly or indirectly via a number of cytokine networks.

The mechanism of action of IGFs has been discussed in Chapter 1 (section 1.3.1) and involves interaction with specific receptors and activation of downstream signalling pathways via PI3 kinase (Kennedy *et al.,* 1997, Marte and Downward, 1997). Recent research from several laboratories has indicated that a signalling pathway from PI3 kinase to the serine/threonine protein kinase PKB (Akt) may mediate protection from apoptosis (Franke *et al.,* 1997, Songyang *et al.,* 1997). Activation of this pathway appears to be able to prevent apoptosis induced by a variety of cellular challenges. The exact mechanisms for the suppression of apoptosis by cell survival factors are unknown, but recent evidence suggests PKB may cause modifications of Bcl-2 proteins. In particular, Bad, a pro-apoptotic member of the family (Kelekar *et al.,* 1997), is phosphorylated by PKB at two sites (Franke *et al.,* 1997). This modification has been shown to prevent Bad from binding to Bcl-XL, thereby inactivating an anti-apoptotic family member (Kelekar *et al.,* 1997, Reed, 1997).

8.4.4.3 Regulation of Bcl-2 family members

The regulation of Bcl-2 family members and its role in cell survival has been discussed in Chapter 1 (section 1.3.3). However, little is known about the regulation of Bcl-2 family members or other components of the apoptosis pathway by nongenotoxic carcinogens. In particular, it is not known whether post-translational modifications of Bcl-2 family members may occur in response to treatment with nongenotoxic carcinogens. Although differential regulation of Bcl-2 family members has been observed during liver regeneration, normally the steady state transcript abundance of different family members is regulated

post-transcriptionally and in part by mRNA stability (Kren *et al.,* 1996, Tzung *et al.,* 1997). The uncoupling of mRNA and protein levels for different Bcl-2 family members observed in hepatocytes treated with the PP clofibrate, suggests that translational or post-translational controls of different family members may regulate survival in the liver (Kren *et al.,* 1996). Post-translational modifications of Bcl-2 family members may be a mechanism by which cytokine-regulated pathways and survival pathways communicate with the apoptosis execution machinery.

8.5 SUMMARY

Nongenotoxic carcinogens cause tumours in rodent bioassays without damaging DNA. Although tumours of the thyroid, skin and kidney can occur, the liver is the major target organ in rats and mice for nongenotoxic carcinogenesis. Dioxin and PB are potent promoters of rat and mouse liver tumours but the largest and most chemically diverse family of nongenotoxic hepatocarcinogens are the PPs. The carcinogenic potential of nongenotoxic carcinogens arises from their ability to perturb growth regulation in their target tissue. PPs mediate their biological responses via activation of the nuclear hormone receptor PPARa which mediates transcriptional regulation of the genes associated with response to PPs. However, the genes that regulate the growth changes associated with hepatocarcinogenesis are unknown. Although suppression of apoptosis and induction of S-phase are likely to play a key role in the carcinogenicity process, recent data support a role for hepatic cytokines such as TNF-α in modulating hepatocyte survival and apoptosis pathways.

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CHAPTER 9

Toxic Mechanisms Mediated by Gene Expression

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9.1 Introduction
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9.1 INTRODUCTION

The transcriptional activation of genes that regulate apoptosis plays a pivitol role in interpreting cellular damage by toxicants and determining the response of a cell or tissue (Figure 9.1). A toxicant can elicit changes in a cell directly by activating gene expression or may act indirectly to perturb pathways associated with cell survival.

Gene transcription is a complex process that requires DNA-dependent RNA polymerase II to bind at the transcription start site within specific promoter sequences. The promoter is located 'upstream' of the coding region of a gene and contains a number of short and specific DNA sequences. The efficiency of transcription is dictated both by the efficiency of polymerase binding and by the rate of progress along the gene to be transcribed. The former is affected both by the accessibility of the DNA and by a complex array of co-factors. Some of these are transcription factors that have their own specific binding sites within the promoter region. These may enhance or repress transcription by promoting or blocking, respectively, the binding to DNA of the key components of the transcription initiation complex. Transcription factors frequently require activation by a specific ligand. This chapter will consider how toxicants modulate apoptosis by altering gene expression and will cover both those toxicants with adverse effects and those claimed to have benefits.

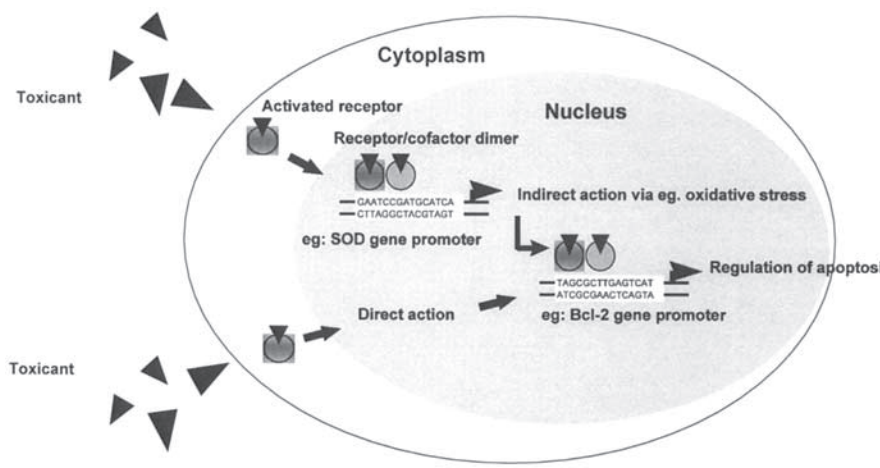

Figure 9.1 Perturbation of apoptosis by toxicants: relevance of gene expression. A toxicant can activate specific receptors, triggering entry to the nucleus and binding to promoter regions of target genes. The promoter is located 'upstream' of the coding region of a gene and contains a number of short, specific DNA sequences that recognize and bind to gene regulatory proteins or transcription factors. Activated genes may be involved directly in the regulation of apoptosis such as the survival gene, Bcl-2, or indirectly involved via a controlling mechanism such as superoxide dismutase (SOD).

9.2 TOXIC MECHANISMS MEDIATED BY GENE EXPRESSION

9.2.1 Oxidative damage

Oxidative damage is known to be a key regulator in chemical-induced apoptosis. It also causes DNA damage which may lead to the development of a number of disease states including cancer, diabetes, atherosclerosis and Alzheimers disease (Breimer and Schellens, 1990). Although there is considerable evidence to implicate oxidative damage in the regulation of apoptosis, little is known of its mode of action. Cells and tissues are subjected to oxidative stress when the concentration of reactive oxygen species (ROS) exceeds the antioxidant capability of the cell (Sies, 1997). This occurs following either an increase in free radicals or peroxides such as H_2O_2, or a decrease in antioxidant levels in the cell (Trush and Kensler, 1991). ROS levels can be modulated by changes in endogenous cytochrome P450s or exogenously following the administration of drugs, hormones, xenobiotics and toxicants (Trush and Kensler, 1991). Oxidative damage can induce apoptosis following an increase in ROS (Buttke and Sandstrom, 1994, Suzuki *et al.,* 1997). Examples of this have been demonstrated in lung epithelial (Janssen *et al.,* 1997) and in human fibroblast cell lines (Gansauge *et al.,* 1997). In addition, antioxidants have been shown to inhibit dexamethasone- and etoposide-induced apoptosis in rat thymocytes (Wolfe *et al.,* 1994).

The molecular pathways that regulate apoptosis provide a possible target for oxidative damage, suggesting a role for transcriptional regulation of genes implicated in cell survival. One such group of genes are members of the family of mitogen activated protein kinases (MAPKs) which play a pivitol role in regulating changes in cell survival and proliferation. This family includes the extracellular signal-regulated protein kinases (ERKs) and the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) (Robinson and Cobb, 1997). Evidence shows that oxidative damage can activate and suppress the MAPKs since H_2O_2-induced apoptosis of HeLa cells is preceded by the inhibition of ERK and the activation of JNK/SAPK (Wang *et al.,* 1998). ERK activation also has an important role in protecting cardiac myocytes from apoptotic death following oxidative damage (Aikawa *et al.,* 1997). However, in cultured mouse keratinocytes, ERKs can be induced by treatment with H_2O_2 and butylated hydroxytoluene hydroperoxide indicating that cell type is an important factor in determining which signalling pathways are modulated (Guyton *et al.,* 1996a,b). It is thought that the dynamic balance between the ERKs and JNK/SAPK pathways is important in determining whether a cell survives or undergoes apoptosis (Xia *et al.,* 1995).

A number of other signalling proteins are involved in ROS-induced apoptosis. The anti-apoptotic protein Bcl-2 is normally down-regulated by oxidative damage and its activation can protect cells against apoptosis following treatment with H_2O_2 (Wang *et al.,* 1998). The pro-apoptotic genes p53 and p21WAF1/CIP1 accumulate during ROS-induced apoptosis in human fibroblast cells (Gansauge *et al.,* 1997). More recently the

protein p85, a regulator of the signalling protein phosphatidyl-3-OH kinase (PI3kinase), has been shown to participate in p53-mediated apoptosis following oxidative damage (Yin *et al.,* 1998). The transcription factor NF-κB, which has been implicated in suppression of apoptosis and survival in some cell types, is also activated by ROS (Schreck *et al.,* 1991, 1992, reviewed in Sen and Packer, 1996). Cells overexpressing the H_2O_2-detoxifying enzyme catalase are unable to activate NF-κB whereas overexpression of Cu/Zn-dependent superoxide dismutase (SOD), which enhances the production of H_2O_2 from superoxide, increases NF-κB activation (Schmidt *et al.,* 1995). In summary, oxidative damage can elevate apoptosis, accompanied by changes in the expression or activation of pro- and antiapoptotic genes. However, a clear role has been demonstrated with only a limited number of genes to date.

9.2.2 Detoxification by glutathione (GSH)

Glutathione (γ-glutamylcysteinylglycine, GSH) has multiple functions in detoxification and its depletion has been associated with an increased risk of chemical toxicity (Kearns and Hall, 1998). GSH can protect cells against toxic insults such as xenobiotics, radiation and infection and has been implicated in drug resistance. GSH also protects against oxidative stress by acting as an antioxidant and many reports suggest that GSH plays a role in apoptosis. For example, depletion of antioxidants such as GSH have been shown to induce apoptosis (Slater *et al.,* 1995, Burdon *et al.,* 1996) and elevation in the levels of antioxidants or overexpression of GSH-dependent enzymes can inhibit apoptosis (Hockenbery *et al.,* 1993, Iwata *et al.,* 1997). There is some debate as to whether apoptosis is triggered by a fall in GSH levels, or an increase in ROS, or both. A decrease in intracellular GSH concentration precedes the onset of apoptosis in murine thymocytes (Beaver and Waring, 1995) whereas GSH depletion is not sufficient to induce apoptosis in U937 human monocytic cells (Ghibelli *et al.,* 1995). In addition, Fas/APO-1 induced apoptosis in Jurkatt cells is associated with the removal of GSH from the cell, which suggests that in this case apoptosis results from a loss in GSH protection rather than an increase in ROS itself (Van den Dobbelsteen *et al.,* 1996). The search for a definitive role for GSH in apoptosis is gaining more momentum particularly with the suggestion that it might have a direct and fundamental role in the activation or inhibition of apoptosis regulatory proteins, such as Bcl-2 (Kearns and Hall, 1998).

9.2.3 DNA methylation

Changes in the methylation status of DNA have been linked with the regulation of apoptosis. The methylation of the C5 position of cytosine (5MC) in 5'-CpG-3' dinucleotides of mammalian DNA is involved in tissue specific gene expression, cellular differentiation and the developmental expression of numerous genes (Cedar and Razin, 1990). The

methylation status of a gene usually shows an inverse correlation with gene expression as hypermethylation usually results in gene inactivation (Baylin, 1997, Baylin *et al.,* 1998). In rat glioma C6.9 cells, 1,25-dihydroxyvitamin D3-induced apoptosis is suppressed by exposure to the DNA demethylating agents 5-azacytidine (5-AzaC) and 5-aza-2'-deoxycytidine (Canova *et al.,* 1998). The authors suggest that this change in DNA methylation could suppress apoptosis through the expression of previously hypermethylated genes with apoptotic suppressing activity. Likewise, in primary murine B cells, unmethylated CpG motifs protect CD40L-stimulated B cells from apoptosis mediated by a Fasspecific antibody (Wang *et al.,* 1997a). However other findings have suggested the opposite since rapid apoptosis is seen in human breast cancer cell lines following administration of 5-aza-2'-deoxycytidine (Ferguson *et al.,* 1997) and in PC12 cells following 5-Azacytidine (Hossain *et al.,* 1997). This would suggest that toxicant-induced changes in DNA methylation might alter the activity of apoptosis related genes.

9.3 PERTURBATION OF APOPTOSIS BY TOXICANTS

A number of toxicants can alter the rate of apoptosis (Table 9.1). For example, the carcinogenic transition metals, chromium, cadmium and nickel are capable of causing an increase in apoptosis along with DNA base modifications, strand breaks and rearrangements. The polycyclic aromatic hydrocarbons (PAHs) also cause apoptosis as well as impairments in carcinogenicity and development. In addition, nicotine and alcohol perturb apoptosis and are closely tied to the development of lung and liver cancers. Although apoptosis is modulated by these compounds, the molecular mechanisms that underpin the changes remain unknown.

9.3.1 Toxic metals

Evidence shows that the transition metals can cause changes in apoptosis. Chromium consistently induces apoptosis in many cell types including Chinese hamster ovary (CHO) cells (Blankenship *et al.,* 1994, Shimada *et al.,* 1998) and cadmium can induce apoptosis in mouse liver cells (Habeebu *et al.,* 1998). However the effects of cadmium-induced changes in apoptosis are inconsistent among various culture systems (Ishido *et al.,* 1995) and has been recently shown to block chromium-induced apoptosis (Shimada *et al.,* 1998). The effects of cadmium or chromium on apoptosis-related gene expression are not well documented although cadmium has been shown to regulate expression of a number of cytokines and immediate-early genes (Beyersmann and Hechtenberg, 1997). Other toxic metals that perturb apoptosis include nickel (II) compounds that are associated with renal and lung cancers in humans and rodents (Ottolenghi *et al.,* 1975, Kasprzak *et al.,* 1994). Recent findings suggest that nickel can arrest growth and induce a dose-dependent increase in apoptosis in CHO cells without affecting the levels of p53 (Shiao *et al.,* 1998).

TABLE 9.1
Summary of changes in apoptosis, oxidative damage, glutathione (GSH), DNA methylation and gene expression/activation caused by toxic or protective compounds

Compound	Apoptosis	Oxidative damage	GSH	DNA methylation	Gene expression/activation	
					Increase	Decrease
Toxic						
Chromium	↑[1]	+	+	?[2]	catalase GSH	
Cadmium	↓↑	+	+	?	c-myc, *c-fos*, p53	
Nickel	↑	+	+	+		
BAP	↓↑	+	+	?	caspase-3, Bcl-2, JNK	c-myc
TCDD	↓↑	+	+	+		
DMBA	↑	+	+	?		
Nicotine	↓	+	+	?	ERK-2, Bcl-2, PKC	
Alcohol	↑	++	+	+	TNF-α, IL-6, IL-1	IGF, IGFR
Preventative						
Butyrate	↑	+	+	?	BAK, c-myc, p21, Bcl-2	Bcl-2, Bcl-XL, JAK-1
Selenium	↑	+	+	+		GSH
GTPs	↑	+	+	?	ERK, JNK, *c-jun*, *c-fos*	
Omega-3 PUFAs	↑	+	+	?		TNF-β, IL-2
Omega-6 PUFAs		+	+	?		

1 Arrows represent an increase or decrease in apoptosis. +represent reported activation.
2 Question mark implies not reported. IGF: insulin-like growth factor; IGFR: insulin-like growth factor receptor; PKC: protein kinase C; IL-1: interleukin-1; IL-6: interleukin-6; JNK: *c-jun* N-terminal kinase; ERK: extracellular signal-regulated protein kinase; TNF-α: tumour necrosis factor alpha; JAK: Janus kinase.

Conversely, mutations in p53 have been found in human cells exposed to nickel (Fritsche *et al.,* 1993).

One mechanism by which these metals may cause toxicity is by oxidative damage following an increase in ROS. This follows the observation that the chemistry and type of mutation that results from metal-induced damage is similar to oxidative attack on DNA (Kasprzak *et al.,* 1995). Both nickel- (Robison *et al.,* 1982, Kasprzak *et al.,* 1990) and cadmium-induced oxidative damage (Snyder, 1988) have been demonstrated in cultured cells and experimental animals. More recently, using the frequency of DNA strand breaks as a marker, both nickel and cadmium were found to cause oxidative damage in human HeLa cells (Dally and Hartwig, 1997). In rat, nickel acetate causes oxidative damage in the kidney and liver although only renal tumours develop; this may be because the liver can support faster DNA repair compared with the kidney (Kasprzak *et al.,* 1992, Kasprzak *et al.,* 1997).

Other toxic metals such as lead and iron have been shown to possess carcinogenic activity and cause oxidative stress and damage. Iron is hepatocarcinogenic in rats and has been shown to modulate ROS production in hepatocytes (Abalea *et al.,* 1998). Lead causes generation of reactive oxygen species and functional impairment in rat sperm (Hsu *et al.,* 1997) and inhibits NO production *in vitro* by murine splenic macrophages (Tian and Lawrence, 1995, 1996). These authors have also shown that cadmium and mercury can inhibit NO production similar to lead but nickel and cobalt had a stimulatory effect (Tian and Lawrence, 1996). Copper, not iron, enhances the apoptosis-inducing activity of antioxidants in human promyelocytic leukemic cells (Satoh *et al.,* 1997). To conclude, an increase in oxidative damage (ROS) results from metal treatment in a number of human and rodent models. It follows that an increase in ROS could mediate the changes associated with the induction of apoptosis by these metals.

Metal toxicity has also been associated with GSH production. GSH modifies the toxicity of a number of metals (for review see Gochfeld, 1997) such as cadmium (Tang *et al.,* 1998), nickel (Lynn *et al.,* 1994) and chromium (Little *et al.,* 1996). It is possible that metals might suppress or remove the protective effect of GSH, causing cells to become more susceptible to apoptosis-inducing agents.

Like GSH, metallothionein (MT), a cysteine-rich metal binding protein could also be involved in co-ordinating the signals from metal toxicants to trigger apoptosis. Although the physiological function of MT is not entirely understood, induction of MT has been observed to be associated with protection from heavy metal toxicity and also cellular resistance to cytotoxic anti-cancer drugs (Klaassen and Liu, 1998). It is believed that it acts as a free radical scavenger and protects against oxidative damage. Indeed, MT-1 transgenic mice, which have a 10-fold higher concentration of MT in their liver than control mice, are resistant to cadmium-induced lethality and hepatotoxicity (Klaassen and Liu, 1998). Enhanced expression of MT in cells induces anti-apoptotic effects but a there is increased susceptibility to apoptotic cell death in MT null cells (Adbel-Mageed and Agrawal, 1997, Kondo *et al.,* 1997). Subsequent work has indicated that the activation of NF-κB may play a role in mediating the anti-apoptotic effects of MT (Adbel-Mageed and Agrawal, 1998). In addition, MT may be linked with the suppression of apoptosis via its antioxidant properties or via a more direct route by interaction with Bcl-2.

Finally, there is increasing evidence for alterations in DNA methylation in metal toxicity, particularly with nickel. Nickel-induced DNA condensation and hypermethylation lead to epigenetic silencing of a transgene in G12 cells (Lee *et al.,* 1995a) and several other gene sequences, including those for thrombospondin and a heme-containing peroxidase, are silenced in nickel-transformed cells (Salnikow *et al.,* 1994). More recently, nickel has been shown to inhibit cytosine 5-methyltransferase activity *in vivo* and *in vitro* as well as elevate total genomic DNA methylation levels (Lee *et al.,* 1998). Therefore it is possible that nickel, and other toxic metals, might perturb apoptosis by direct modulation of genes that regulate cell survival. Although the effects of metals on the methylation status

of apoptotic genes has to be determined, it would present informative data on the mode of action with regard to apoptosis.

9.3.2 Polycyclic aromatic hydrocarbons (PAHs)

Benzo(a)pyrene (BaP) induces apoptosis and up-regulates the cysteine protease caspase-3 (ICE/Ced-3) in mouse Hepa cells (Lei *et al.,* 1998). BaP also caused a time and dose-dependent activation in JNK1 activity indicating that stress-activated kinase activation is involved in BaP-induced apoptosis (Lei *et al.,* 1998). BaP also induces cell death in mouse epidermis (Miller *et al.,* 1996) but, in contrast, can inhibit apoptosis in Syrian hamster embryo cells (Dhalluin *et al.,* 1997). In this latter experiment, Bcl-2 was up-regulated and levels of the Bcl-XL protein did not change, suggesting that inhibition of apoptosis via Bcl2 might contribute to the early stages of the carcinogenic process.

Dioxin (2, 3, 7, 8-tetrachlorodibenzo[p]dioxin; TCDD) can cause changes in the rate of apoptosis although the nature of these changes depends much on the experimental system. For example, TCDD can induce apoptosis in mouse thymocytes *in vivo* but not *in vitro* (Kamath *et al.,* 1997). Other reports in immature rat and mouse thymocytes (McConkey *et al.,* 1988, Rhile *et al.,* 1996) show that TCDD can induce apoptosis *in vitro*. Another PAH, dimethylbenz[a]anthracene (DMBA) has also been shown to induce apoptosis in an A20.1 murine B-cell line by a mechanism that is dependent on cellular Ca^{2+} (Burchiel *et al.,* 1993).

The underlying mechanisms of action of PAHs on apoptosis are not clear. It was originally believed that all biological effects of TCDD were mediated through the aryl hydrocarbon (Ah) receptor (DeVito and Birnbaum, 1995), a ligand-activated member of the bHLH-PAS family of transcription factors (Burbach *et al.,* 1992). However, it has been shown recently that TCDD-induced apoptosis in a human leukemic lymphoblastic T cell line was not dependent on the Ah receptor (Hossain *et al.,* 1998). In this case, apoptosis was inhibited by caspase and tyrosine kinase inhibitors and was linked to a rapid increase in JNK and a decrease in Bcl-2 protein levels. The ability of single doses of TCDD to induce oxidative stress in hepatic and some extrahepatic tissues of animals is also well documented (Stohs *et al.,* 1990, Alsharif *et al.,* 1994). TCDD induces oxidative stress in mice as measured by production of superoxide anion, an effect that is controlled in part by the Ah receptor complex (Alsharif *et al.,* 1994). Recent data suggests that BaP also increases oxidative stress in human epidermoid carcinoma cells (Liu *et al.,* 1998). The effects of TCDD on glutathione synthesis has also been documented (reviewed in Schrenk, 1998).

9.3.3 Nicotine and alcohol

Nicotine blocks apoptosis induced by a variety of stimuli such as TNF-α, UV light and a number of chemotherapeutic drugs (Wright *et al.,* 1993). The mechanisms involved in

nicotine-induced suppression of apoptosis remain unclear. Nicotine has no effect on JNK and p38 MAPK activity but can activate ERK-2 which results in an increase in Bcl-2 and a suppression of apoptosis in human lung cancer cells (Heusch and Maneckjee, 1998). The same workers have shown that nicotine can stimulate protein kinase C (PKC) activity in lung cancer cell lines (Maneckjee and Minna, 1994) and can block the inhibition of PKC and ERK-2 activity by anti-cancer agents, such as therapeutic opioid drugs (Heusch and Maneckjee, 1998). It has been suggested that the activation of the nicotine receptors C6 or C10 can lead to a suppression of apoptosis which, in some cases, is mediated via the PKC pathway. The effect of nicotine on oxidative damage and DNA methylation have also been investigated although reports are few. The effects of snuff and nicotine on DNA methylation by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a powerful tobacco-specific N-nitrosamine, have been investigated. NNK induced methylation in rat liver, nasal mucosae and oral cavity, snuff suppressed methylation whereas nicotine had no effect, indicating that nicotine has no effect on the genotoxicity of NKK (Prokopczyk *et al.,* 1987).

The mechanism by which chronic alcohol abuse induces widespread cell and tissue damage is unknown. Some reports suggest that alcohol toxicity is associated with increased levels of endotoxin in plasma (Su *et al.,* 1998). Others report that alcoholic liver injury results from a decrease in oxygen supply which produces hypoxia (Yuki and Thurman, 1980, Nanji *et al.,* 1995). There are also many examples of ethanol-induced apoptosis in hepatocytes and other cell types (Benedetti *et al.,* 1988, Aroor and Baker, 1997, Kurose *et al.,* 1997). In hepatocytes, ethanol administration induces an increase in lipid peroxidation either by enhancing the production of ROS and/or by decreasing the level of endogenous antioxidants (Sergent *et al.,* 1995, Higuchi *et al.,* 1996). In addition, agents that inhibit ethanol-induced oxidative stress effectively attenuate hepatocyte apoptosis and/or necrosis. Therefore, it has been suggested that ethanol-induced hepatocyte apoptosis is driven by an oxidant-dependent mechanism since oxidative stress is recognized to be a key step in the pathogenesis of ethanol-associated liver injury (Higuchi *et al.,* 1996). In support of this hypothesis other findings have shown that ethanol-induced ROS in hepatocytes mediates the DNA fragmentation step of apoptosis and that intracellular antioxidants such as glutathione protect hepatocytes against ethanol-induced apoptosis (Kurose *et al.,* 1997).

In addition to oxidative damage, alcohol causes changes in the expression of apoptosis-related genes. The effects of alcohol on insulin-like growth factor (IGF) and the IGF-1 receptor has been well documented (Resnicoff *et al.,* 1993, 1996). It has been shown that IGF-1 receptor controls the extent of cell proliferation in a variety of cell types and protects cells from apoptosis. Ethanol at low concentrations markedly inhibits IGF-1 receptor autophosphorylation and IGF-1-mediated cell growth. This receptor is likely to play an important role in ethanol-induced apoptosis as it can also act as a strong inhibitor of etoposide-induced apoptosis in BALB/c 3T3 cells (Sell *et al.,* 1995). Up-regulation in

cytokine expression has also been implicated in ethanol induced apoptosis. In sublingual salivary gland cells chronic alcohol ingestion causes enhancement of TNF- α expression and apoptosis (Slomiany *et al.,* 1997). In HepG2 cells ethanol-induced cytotoxicity is accompanied by an increase in TNF-α, interleukin-1α (IL-1α) and interleukin-6 (IL-6) and a reduction in glutathione reductase levels (Neuman *et al.,* 1998).

In summary, both nicotine and alcohol are important toxic regulators of apoptosis. Nicotine suppression of apoptosis appears to involve Bcl-2 and the activation of the PKC pathway whereas alcohol induced apoptosis is regulated primarily through oxidative damage. However the observation that alcohol can inhibit IGF signalling suggests that apoptosis-inducing toxicants may operate by inhibiting survival pathways.

9.4 PERTURBATION OF APOPTOSIS BY PROTECTIVE COMPOUNDS

Compounds with beneficial health effects may act by regulating apoptosis. Not surprisingly, many of these are known dietary supplements. This includes butyric acid or butyrate, selenium, green tea polyphenols (GTPs) and the polyunsaturated fatty acids (PUFAs). Most of these compounds are described as having a health protective role although they can both induce or suppress apoptosis.

9.4.1 Butyrate

Butyrate, in the form of dietary fibre supplementation, leads to reduced colonic cell proliferation and a lower tumour mass (Boffa *et al.,* 1992, McIntyre *et al.,* 1993). It inhibits growth in human hepatocellular carcinoma (HCC) cells (Yamamoto *et al.,* 1998) and induces apoptosis in human colorectal carcinoma cells (Hague *et al.,* 1993, Mandal and Kumar, 1996). In human colonic adenoma cell lines butyrate-induced apoptosis can be associated with either an increase in the levels of the pro-apoptotic gene, Bak, or a reduction of the anti-apoptotic gene Bcl-2 (Hague *et al.,* 1997). Likewise in colorectal carcinoma cells an inverse relationship was found between the levels of Bcl-2 and sensitivity of the cells to undergo apoptosis in response to butyrate (Mandal *et al.,* 1997). Over expression of Bcl-2 results in the suppression of butyrate-induced apoptosis and enhanced cell survival (Mandal *et al.,* 1997). Butyrate has also been shown to down-regulate the levels of Bcl-2 in other cell types including human breast MCF-7 cells (Mandal and Kumar, 1996) and in myeloid leukemic cells (Calabresse *et al.,* 1994). However, more recent evidence has shown that sodium butyrate has no affect on apoptosis in HCC cells where it up-regulates Bcl-2; thus, the mechanism of butyrate-induced apoptosis is not fully understood (Saito *et al.,* 1998).

Butyrate targets a number of other apoptotic genes. In addition to Bcl-2 and Bak, butyrate significantly lowers the levels of the apoptosis antagonist Bcl-XL in human fibroblasts (Chung *et al.,* 1998). In a chronic leukemia cell line the butyrate analogue,

arginine butyrate, inhibits the expression of the anti-apoptotic factor p210 Bcr-abl and other phos-phoproteins including JAK-1 kinase (Urbano *et al.,* 1998). Furthermore, both c-myc and p21 WAF/Cip1 are induced by butyrate, independently of p53 (Hague *et al.,* 1993, Janson *et al.,* 1997, Heerdt et al., 1998).

Butyrate has been shown to be a potential inhibitor of apoptosis in other tissues since it markedly suppresses c-myc-mediated apoptosis in murine plasmacytomas and human Burkitt lymphomas (Alexandrov *et al.,* 1998). Also, butyrate can induce the synthesis of MT in ROS 17/2.8 cells thus protecting against the possible pro-apoptotic effects of oxidative damage (Thomas *et al.,* 1991). In summary, the protective effects of butyrate may be based upon an induction of apoptosis, possibly due to the down-regulation of Bcl-2. However consideration should be given to conflicting reports that show butyrate-induced suppression of apoptosis.

9.4.2 Selenium

Selenium markedly reduces the incidence of lung, colon and prostate cancers (Clark *et al.,* 1996, Harrison *et al.,* 1997). Sodium selenite is one of the most effective selenium compounds and has been shown, like butyrate, to induce apoptosis in human colonic carcinoma cells (Stewart *et al.,* 1997). Sodium selenite is metabolized to hydrogen selenide through its reduction to glutathione and this is thought to be an important step in exerting its anti-proliferative and apoptotic effect. Selenodiglutathione (SDG), the primary metabolite of selenite reduction to glutathione, also has potent anti-carcinogenic and cytotoxic properties (Lanfear *et al.,* 1994). Like selenite, SDG has been shown to induce apoptosis in a number of mammary carcinoma cell lines (Lanfear *et al.,* 1994, Thompson *et al.,* 1994) although the biological mechanisms underlying these effects are unknown. Apoptosis may result from H_2O_2-induced oxidative stress since H_2O_2 is a by-product of selenite reduction to SDG and the role of oxidative damage as a pro-apoptotic factor has been well documented. Selenomethione, another selenium compound and primary constituent of dietary selenium supplement, has anti-carcinogenic properties that have been demonstrated in both lung and colon tumour cell lines (Redman *et al.,* 1997). Selenomethione induced apoptosis and decreased the polyamine content in these cancer cell lines suggesting that the anti-carcinogenic effects of selenium supplementation might be due to depletion of polyamine levels. Polyamines, such as spermine and spermidine, are required for normal cell proliferation and development but it appears that imbalances in the metabolism of polyamines may constitute a signal for apoptosis.

9.4.3 Tea polyphenols

Green tea polyphenols (GTPs), major constituents of green tea, are potent chemopreventative agents in a number of animal experimental models of cancer. Feeding

of GTP has been shown to suppress skin tumourigenesis induced by both aromatic hydrocarbons and UV light and inhibit tobacco induced lung tumourigenesis in mice (Wang *et al.,* 1991, Katiyar *et al.,* 1993a, b, Shi *et al.,* 1994).

Around 10% of the dry weight of green tea is made up of catechins, of which (-)-epigallocatechin-3-gallate (EGCG) is thought to be an active anti-cancer component (Balentine, 1992). However, during the manufacture of black tea the catechins are oxidized and polymerized to form the black tea polyphenols, theaflavins and thearubigins (Balentine, 1992). These compounds have antioxidative activities, due to their radical scavenging and metal chelating functions, and are also thought to have tumour inhibitory activities (Serafini *et al.,* 1996).

Several lines of evidence suggest that the induction of apoptosis may explain the growth inhibitory properties of tea extracts. An induction in apoptosis and concomitant fall in DNA synthesis has been observed in human stomach cancer KATO III cells following exposure to green tea catechin extract and EGCG (Hibasami *et al.,* 1998). Likewise green tea has been shown to cause apoptosis and cell cycle arrest in a number of other human carcinoma cell lines (Ahmad *et al.,* 1997, Zhao *et al.,* 1997, Katdare *et al.,* 1998). Also exposure of a lung tumour cell line H661 to either EGCG or theaflavins led to an increase in apoptosis as well as a growth inhibition (Yang *et al.,* 1998). These workers have also shown that tea polyphenols induce production of H_2O_2 which may mediate this apoptosis.

Other evidence suggests that the protective effects of GTP are due to the inhibition of enzymes, such as cytochrome P450, involved in the bioactivation of carcinogens (Bu-Abbas *et al.,* 1994a,b). Reports indicate that GTP causes increases in the activities of the glutathione S-transferases (Khan *et al.,* 1992, Lee *et al.,* 1995b). Tea and tea polyphenol compounds have been shown to inhibit the activities of a number of other important regulatory enzymes including protein kinase C, ornithine decarboxylase and DNA topoisomerase II (Austin *et al.,* 1992, Jankun *et al.,* 1997). Further studies have shown that the mitogen-activated protein kinases ERK-2 and JNK-1 are strongly activated by GTPs as are the immediate-early genes c-*jun* and c-*fos* (Yu *et al.,* 1997). These workers have suggested that the stimulation of MAPKs by GTP may provide the early signalling events that lead to the eventual activation of Phase II detoxifying enzymes. However despite an increasing list of GTP targets, a mode of action is still to be elucidated.

9.4.4 Dietary fats

Epidemiological and experimental studies indicate a strong relationship between dietary fats such as polyunsaturated fatty acids (PUFAs) and cancer. Dietary fat can be both beneficial or detrimental to animals. For example, corn oil which contains high levels of omega-6 PUFAs, such as linoleic acid, enhances chemically-induced colon tumourigenesis in rodents, whereas fish oil, which is rich in omega-3 PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) reduces colon carcinogenesis (Torosian *et al.,* 1995).

In rat, the protective effects of fish oil against experimentally induced colon tumorigenesis results from an increase in apoptosis and differentiation, rather than a decrease in proliferation (Chang *et al.,* 1998). In addition, the the anti-tumour effect of DHA corresponds with its induction of apoptosis (Calviello *et al.,* 1998). In contrast the anti-tumoural effects of EPA appears related mainly to inhibition of cell proliferation (Calviello *et al.,* 1998). Other Omega-3 PUFAs inhibited growth by inducing apoptosis in three pancreatic cancer cell lines and in a HL-60 leukaemic cell line (Hawkins *et al.,* 1998). In this latter study apoptosis was preceded by lipid peroxidation indicating that apoptosis might be caused by an oxidative mechanism.

Conversely, the omega-6 PUFAs, such as linoleic acid, enhance tumour growth not only by promoting cell proliferation but also by suppressing apoptosis (Tang *et al.,* 1997). Although NO production is inhibited by the anti-tumourigenic omega-3 PUFAs, probably by suppression of NO synthetase, no inhibition is observed with omega-6 PUFAs such as linoleic and oleic acid or the saturated fatty acid, stearic acid (Ohata *et al.,* 1997).

To conclude, these protective compounds share the ability of detrimental toxicants to induce apoptosis. However, the apoptosis induced by the protective compounds may remove unwanted or deleterious cells from the target tissue. Evidence for changes in apoptosis related gene expression is inconsistent, although in many cases, down-regulation of *bcl-2* precedes induction of apoptosis. Again, oxidative damage, GSH and MT are also potential mediators of changes in apoptosis caused by these compounds.

9.5 SUMMARY

Toxicants may have adverse or beneficial effects on health in humans and in animal models. Some toxicants can suppress apoptosis (see Chapter 8) although examples of an induction of apoptosis are more numerous. There are a variety of mechanisms implicated in the regulation of apoptosis by toxicants, including changes in the expression or activation of specific genes that regulate cell survival and apoptosis (Table 9.1). However, increases in ROS (oxidative damage) and a decrease in GST production are frequently observed, suggesting that the effects on gene expression may be indirect. Toxicant-induced changes in the methylation status of apoptosis-related genes may present some new leads. In addition, alcohol toxicity suggests that toxicants may cause the suppression of survival pathways, mediated by IGFs. The study of toxicant-induced gene expression should provide a mechanistic explanation for the regulation of apoptosis by toxic and protective compounds.

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CHAPTER 10

Detection and Biomarkers of Apoptosis

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10.1 Introduction
10.2 Parameters affecting the detection of apoptosis
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10.1 INTRODUCTION

Apoptosis, as a mediator of the action of toxicants, has been described in the preceding chapters. In addition, the pathways that regulate apoptotic cell death have been considered in detail both at the cellular and at the molecular level. However, a clearer understanding of the role of apoptosis in mediating any biological process depends upon accurate quantitation. This chapter will describe the morphology and biomarkers of apoptosis and will consider the application of such knowledge to the quantitation of cell death *in vivo* and *in vitro*.

The first description of the morphology of apoptosis involved stimulation of thymocytes with glucocorticoids (Arends and Wyllie, 1991). Subsequently, it became apparent that the morphological features described were common to many other tissues such as the kidney (Chapter 6) and liver. In whole tissues or for adherent cells in culture, the cell assumes a rounded appearance and a clear 'halo' appears as the cell becomes separated from its neighbours (Figure 10.1A). Using classical histological stains (Kerr *et al.,* 1972), the nucleus becomes deeply stained with haematoxylin as the chromatin condenses and marginates and the cytoplasm becomes deeply eosinophilic. This series of morphological changes is represented diagramatically in Figure 10.1B.

10.2 PARAMETERS AFFECTING THE DETECTION OF APOPTOSIS

As discussed in Chapter 1, the biochemical stages of apoptosis can be described as imposition of damage, sensing, coupling and execution. The majority of techniques available detect the cellular changes associated with the final, execution phase of apoptosis rather than any of the latent phases of signalling and commitment. As such, all methodologies underestimate the number of apoptotic cells present. Other factors affecting success in quantitating apoptosis include the tissue to be studied and the marker chosen (see Table 10.1). In addition, some stages within execution are more readily detected than others; for example, the crescentic, marginated chromatin is far more easily recognized than the following stages where nuclear disintegration has occurred (Clarke, 1990). Different tissues in the vertebrate body display different rates of apoptosis depending upon their function. Those tissues which display high rates of cell replication such as the haemopoietic tissue and GI tract generally show higher rates of apoptosis than those tissues, like the liver, where cell replication rates tend to be low. Tissues with a small proportion of apoptotic cells will present the researcher with additional problems from those with high rates of apoptosis. Techniques involving homogenization of the whole tissue, such as ELISA assays, may dilute out the small proportion of apoptotic cells to such an extent that they fall below detection limits (Naruse *et al.,* 1994, Kawarada *et al.,* 1998).

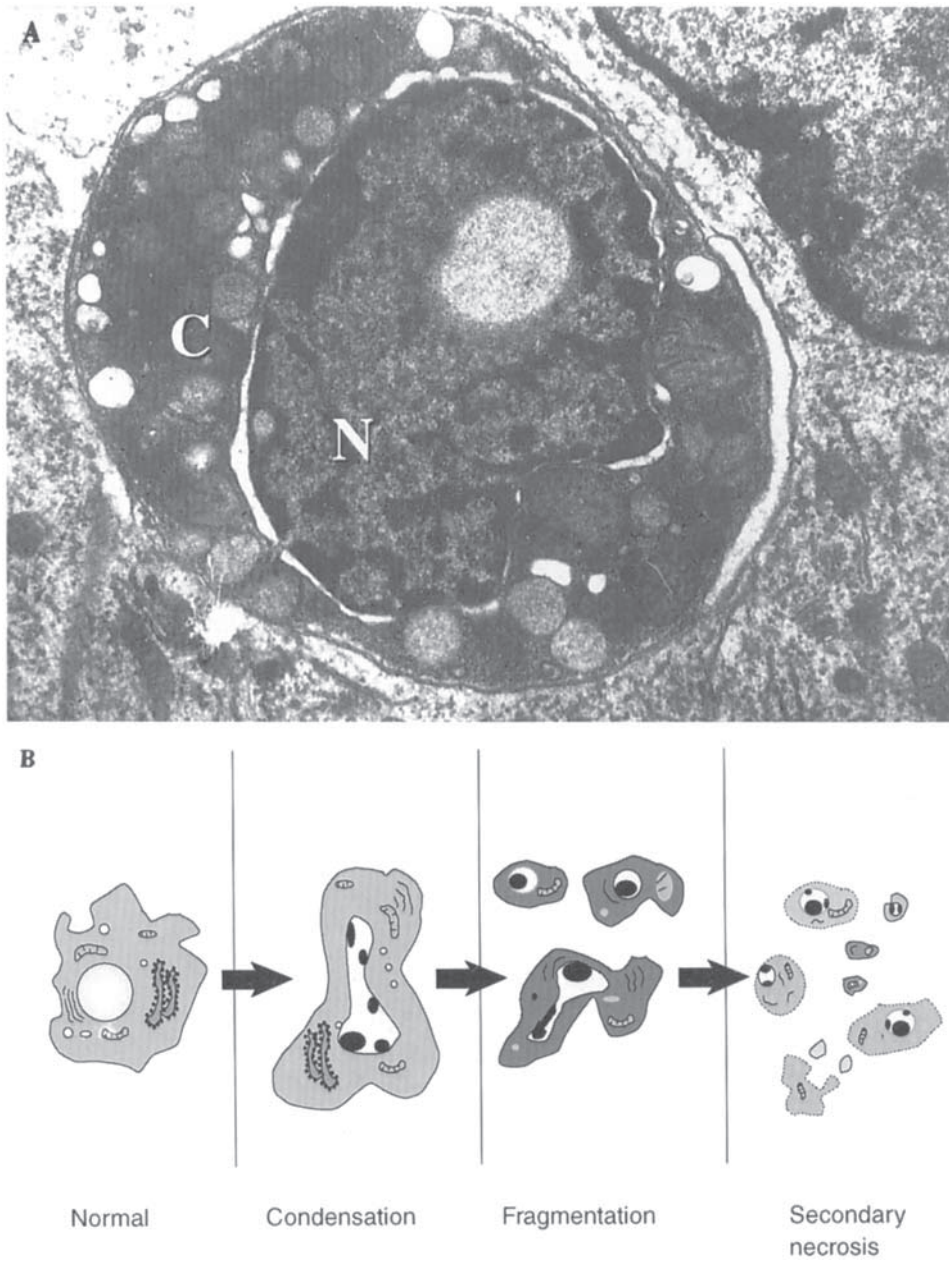

Figure 10.1 (A) Electron micrograph of an apoptotic heptocyte. The cell has rounded off and lost contact with its neighbours. It shows condensed cytoplasm (C) and nucleus (N) but still retains recognizable organelles such as mitochondria. Magnification X 4,750. (B) Sequence of morphological events that occur as a cell becomes apoptotic. The end stage of secondary necrosis normally takes place after the cell fragments have been phagocytosed by other cells.

TABLE 10.1
Potential marker molecules for apoptotic studies

Marker	Specificity for apoptosis	Change	Reference
c-myc/fos	Not specific	↑	Buttyan *et al.*, 1988
Bcl-2/Bax family	Good	↓/↑	Vaux *et al.*, 1988
Ca^{2+}	Not specific	↑	Kyprianou and Isaacs, 1988
DNA fragmentation	Good	↑	Gavrieli *et al.*, 1992
p53	Limited	↑	Yonish-Rouach, 1996
Morphology	Very good	Condensation/ fragmentation	Bursch *et al.*, 1986
Hoechst 33342	Good?	Condensation of DNA	James and Roberts, 1996
Transglutaminase	Good	↑	Fesus *et al.*, 1987
Caspases	Good	↑ (Enzymatic activity)	Gagliardini *et al.*, 1994

Similarly, histological techniques using haematoxylin and eosin on tissue sections may detect a single apoptotic cell present within a tissue section but quantitation will require considerable effort and operator skill.

ELISA assays provide quantitative data following tissue homogenization and differential centrifugation of the cellular components. However it is difficult to relate figures obtained by ELISA assay back to changes in cell populations since a knowledge of the cellular content of the marker in question is required. Similarly, DNA laddering has been regarded as a definitive marker of apoptosis but there is a question regarding whether the phenomenon occurs in all instances of apoptosis (Cohen *et al.*, 1994), and in situations where only a small proportion of the cells undergo apoptosis its lower level of sensitivity may preclude it detecting the increase due to the dilution factor of a large amount of DNA from non-apoptotic cells.

Histological methods can be sensitive, depending upon the marker employed (see Table 10.1), and are applicable to almost all tissues requiring study. However the effort involved in assessing histological preparations of tissues in a quantitative way is always going to be considerable even though their accuracy may be great (Bursch *et al.*, 1986).

Frequently, there may be a compromise between specificity and ease of detection. A marker which is not specifically expressed by apoptotic cells may still be useful if it allows an investigator to distinguish the apoptotic cell from its neighbours (McCarthy and Evan, 1998). An example of such a marker is haematoxylin and eosin, in routine histological preparations, and Hoechst 33342 on cells in culture. Neither stain specifically localizes apoptotic cells but, because they both differentially stain the apoptotic cells, they are of real use.

10.3 METHODS FOR DETECTING APOPTOTIC CELLS

Methods for detecting apoptosis can be divided into three broad categories based upon the methodology for tissue preparation. The first involves processing of intact tissue for histology and then utilizing specific or non-specific markers of apoptotic cells. The second involves the preparation of individual cells from the intact tissue and the third method involves disruption of the tissue and cells and analysis of the released cellular proteins and/or DNA.

10.3.1 Methods using intact tissue

Tissues can be fixed either by chemical fixatives or by freezing. By definition, these methods involve minimal disruption of the tissue and are able to give precise spatial information regarding the distribution of apoptotic cells within a tissue or organ. Following fixation and sectioning, the choice of histological methodology falls broadly into morphological recognition of the cells by microscopy, immunocytochemical detection using antibodies raised against a molecule expressed by cells undergoing apoptosis or detection of the DNA fragmentation occurring during the apoptotic process. Each of these will be discussed separately.

Morphological recognition of apoptotic cells by either light or electron microscopy remains a widespread technique. With training, the cells can be recognized accurately and may be counted using morphometric techniques on a sufficient number of tissue sections to provide a representative sample. Advantages include ease of use since these morphological techniques are compatible with routine histological or ultrastructural examination. The main disadvantages arise from the subjectivity of the recognition of apoptotic cells which may depend upon the experience of the operator. The studies of Bursch *et al.* (1986) have incorporated counts of apoptotic cells both with and without nuclear fragments, with the identification of the latter being purely on the basis of round eosinophilic bodies, most often contained within other unaffected cells (Bursch *et al.,* 1994). Thus, inter-operator and inter-laboratory variability must be managed to avoid inaccuracies. In addition, judgement of sampling size both within the section and between sections of the same biological sample must be determined statistically to limit variability.

Light microscopy of apoptotic cells (Figure 10.2) has advantages in that the preparation is rapid and inexpensive and requires minimal technical input. In addition, the numbers of apoptotic cells in each section are sufficient for quantitation; this can be automated using similar technology to that described previously for routine evaluation of cell proliferation indices (Soames *et al.,* 1994).

In addition to light microscopy, electron microscopy can be applied to identify apoptotic cells by their morphology. This methodology probably represents the 'gold standard' as far as apoptosis in intact tissue is concerned since the apoptotic bodies may be

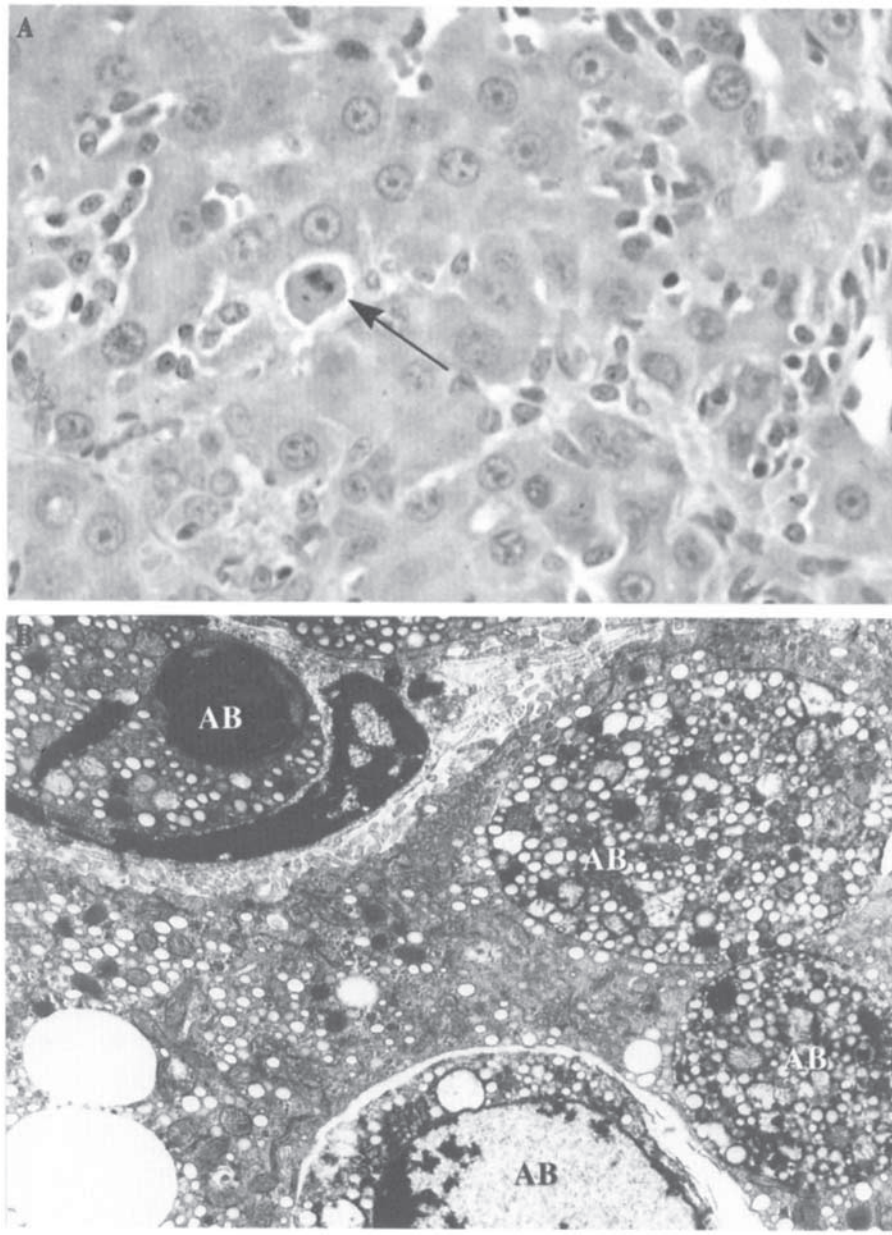

Figure 10.2 (A) Histological appearance of an apoptotic cell in the liver of a rat. Haematoxylin and eosin stained, X 380 magnification. (B) Electron micrograph of a number of apoptotic bodies (AB), some with and some without nuclei. These are present within both hepatocytes and within Kupffer cells in the liver of a mouse given a chlorinated solvent. In all cases recognizable cytoplasmic organelles are present with a similar morphology to those present within the non-apoptotic hepatocytes that have engulfed the cells. X 2,375 magnification.

identified in all of their stages of progression (Clarke *et al.,* 1990), with or without nuclear fragments (Figure 10.2B).

Immunocytochemistry can be used to detect apoptosis since many proteins are expressed specifically or preferentially by cells undergoing apoptosis. There are several candidate proteins listed in Table 10.1. Transglutaminase is an enzyme involved in the reaction resulting in the condensation of tubular proteins which enables the apoptotic cells to be recognized in tissue sections stained with routine histological stains (Folk 1980, Hager *et al.,* 1997). The transglutaminase-catalyzed cross-linking reaction leads to protein polymerization which is extremely strong (Folk and Finlayson, 1977). The overexpression of tissue or type II transglutaminase is thought to be specific for apoptosis (Piacentini *et al.,* 1994) although low level expression was noted in viable endothelial cells, smooth muscle cells and mesangial cells (Thomazy and Fesus, 1989).

Although marker proteins such as c-myc, p34, SGP-2 and vitronectin have been advocated for use in identifying apoptotic cells (Buttyan *et al.,* 1989, Savill *et al.,* 1990, Dini *et al.,* 1992, Evan *et al.,* 1992, Shi *et al.,* 1994) they are certainly not specific to the process and their use has to be combined with morphology to positively identify the cell as apoptotic. One recent development is the use of caspases (see Chapter 2) as biomarkers of apoptosis. Caspases constitute a family of proteases that are activated at the final, execution stage of apoptosis by cleavage from a pro-form. Activation of the caspase proteolytic cascade results in apoptotic cell death (see Chapter 2). Although antibodies to the caspases are available that can be used for immunocytochemistry, caspase protein levels may appear unchanged since activation results from enzymatic cleavage. Thus, caspase assays based on activity are very useful as biomarkers of apoptosis and will be discussed in section 10.4.3.

With all immunocytochemistry procedures, the fixation methodology must be tailored to suit the tissue and the epitopes (Swanson, 1997). Aldehyde fixatives such as paraformaldehyde or formaldehyde, or alcohol-based fixatives such as methacarn (Oyaizu *et al.,* 1996) are all suitable for immunocytochemistry. In general, the alcohol-based fixatives are less destructive to the antigenic determinants since the aldehyde fixatives introduce extensive cross linking that may render the epitope unrecognizable to an antibody raised against the native protein. In some cases, fixatives may be counter-indicated and tissues would have to be fresh-frozen prior to sectioning. In the case of tissues already fixed, antigen retrieval methodology may have to be applied (Werner *et al.,* 1996). In general, a delicate balance is required between fixation which preserves morphology at the expense of antigenicity; these factors must be balanced for successful identification of apoptotic cells by immunocytochemical techniques.

During apoptosis, DNA fragmentation occurs simultaneously with the morphological changes of chromatin condensation. Several systems have been developed that permit the localization of these DNA breaks in tissue sections (Gorczyca *et al.,* 1993) and in isolated cells in culture (reviewed in Roberts *et al.,* 1995). Overall, the methods fall into

three functional categories dependent on the enzyme used for the tagging procedure. *In situ* end labelling (ISEL) uses DNA polymerase I (Kornberg polymerase) which has dual functionality; it can add digoxigenin (DIG)-labelled bases to recessed free 3' hydroxyl groups but can also nick translate from single stranded breaks, hence the alternative acronym ISNT (*in situ* nick translation). Alternatively, the Klenow fragment of DNA polymerase I (which lacks the nick translation activity of Kornberg) can be used to carry out end labelling of DNA on tissue sections. Alternatively, the terminal deoxynucleotidyl transferase enzyme (Tdt) has been used for Tdt-mediated dUTP nick end labelling (TUNEL) or tailing. This enzyme can add DIG-labelled bases to both double stranded or single stranded 3'-hydroxyl groups. The bound markers on newly synthesized DNA can then be detected by binding to avidin-conjugated peroxidase or other suitable enzyme marker.

The methodology has been criticized as being non-specific since it also stains necrotic cells (Grasl-Kraupp *et al.,* 1995) and gives false positives (Stahelin et al., 1998). However, this would be anticipated since DNA strand breaks are not unique to apoptosis; as with all immunocytochemical techniques, care is needed in interpretation and coincidence of the stain with apoptotic morphology should be used as an additional criterion.

10.3.2 Methods using isolated cells

In many applications the necessity for maintaining tissue organization is secondary to the many advantages offered by isolated cells. For example, cells can be made relatively homogeneous and can be generated in large numbers. In some cases, cells can be synchronized to enter apoptosis in large numbers (Sen *et al.,* 1990) and the temporal aspects of apoptosis can be studied in the same cell as it progresses from normality to secondary necrosis (Pulkkinen *et al.,* 1996). Importantly, the effects of exogenous factors such as calcium (Story *et al.,* 1992), xenobiotics (James and Roberts, 1996) and growth factors such as transforming growth factor-ß (James and Roberts, 1996) can be studied under defined conditions (Wang et al., 1995, Tong *et al.,* 1996).

McCarthy and Evan (1998) utilized time-lapse video microscopy to study the effects of cell density and cell contact on the apoptotic rate and the subsequent phagocytosis of apoptotic cell fragments. Using this system, they demonstrated that survival factors such as Bcl-2 and insulin-like growth factor I suppressed the onset of apoptosis in cells but did not affect the kinetics of individual events. In contrast, inhibitors of caspases had no effect on the onset of apoptosis but prevented each apoptotic death from going to completion (McCarthy *et al.,* 1997). Thus, time-lapse video microscopy can provide unique information about the fates and rates of apoptosis in individual cells. However, the need for specialist equipment can be prohibitive.

The process of apoptosis can be studied by immunocytochemistry in isolated cells using antibodies against a range of apoptosis-associated antigens (Table 10.1) as described earlier for tissue sections (section 10.3.1). Again, operator expertise is essential for accurate

TABLE 10.2
Commonly used DNA stains for morphology and flow cytometry (from Fraker *et al.,* 1995)

DNA binding dye		Laser excitation line (nm)	Emission range (nm)
Propidium iodide	Intercalative	488	550–640
Ethidium bromide	Intercalative	488	550–640
Acridine orange	Intercalative	488	510–530
7-amino-actinomycin D	Intercalative	488, 541	640–680
Daunomycin	Intercalative	488	560–600
Mithramycin A	External	457	510–530
Chromomycin A	External	457	550–580
Olivomycin	External	457, 480–500	530–560
Hoechst 33258	External	351–364	380–420
Hoechst 33342	External	351–364	380–420
DAPI	External	351–364	380–420

identification of apoptotic cells and appropriate fixation methodology is required to achieve optimal immunostaining. In addition, cells in culture have an intact cell membrane which has to be made permeable to allow the entry of the relatively large antibody molecules. This is usually achieved by alcohol fixation or by a combination of exposure to hypotonic saline and freeze-thawing (Farahat *et al.,* 1994). The choice of final reaction product to visualize the apoptotic cells can be a chromogen or a fluorochrome (Heidenreich et al., 1996).

As an alternative to immunocytochemistry, DNA intercalating dyes can be used for detection of apoptosis in isolated primary cells or in cell lines *in vitro*. There are several available such as propidium iodide, acridine orange or Hoescht 33258. These dyes allow detection of condensed DNA within the nuclei of apoptotic cells and provide an important tool in the study of apoptosis *in vitro* (Juan *et al.,* 1996). Under the right conditions very accurate estimations of the number of apoptotic cells present following the treatment of the culture with, for example, growth factors or cytotoxic drugs, can be made (James and Roberts, 1996, McGahon *et al.,* 1998). The TUNEL technology, described for tissue sections in 10.3.1, is also applicable to cells in culture (Gold *et al.,* 1993).

Flow cytometry is probably one of the most efficient methods for the study and quantification of apoptosis (Dive *et al.,* 1992, Telford *et al.,* 1994, van Engeland *et al.,* 1998). As cells pass through a laser beam, they can be differentiated on the basis of their size and density. Using propidium iodide, the DNA content per cell can also be determined to permit analysis of the proportion of cells undergoing apoptosis (Dive *et al.,* 1992). Table 10.2 shows a list of the common DNA dyes used in flow cytometry (taken from

Fraker *et al.,* 1995). Flow cytometry can be especially useful when mixed cell populations need to be analyzed since the technique can differentiate more than one parameter simultaneously. For example, thymocytes can be analyzed simultaneously for DNA content using propidium iodide and cell typed by their expression of CD4/CD8 using antibodies (McCarthy and Evan, 1998). Alternatively, propidium iodide can be used alongside other markers associated with apoptosis such as transglutaminase, annexin V (see 10.3.3), TUNEL (see 10.3.3) or Bcl-2 (Al-Rubeai, 1998).

10.3.3 Methods using subcellular fractions

Many assays use other properties of apoptotic cells such as protein, membrane and DNA changes. There are many kits available commercially that exploit these changes to provide rapid and cost-effective measurement of cell death. Some assays utilize an ELISA (Nilsson, 1989) to detect specific proteins or look for the presence of DNA in inappropriate cellular fractions (Chen *et al.,* 1997, Jaeschke *et al.,* 1998, Kawarada *et al.,* 1998, Wassermann *et al.,* 1998). One method used in the study of apoptosis uses primary antibodies directed either against single-stranded DNA itself (Naruse *et al.,* 1994, Kawarada *et al.,* 1998) or raised against histone protein (Suenaga and Abdou, 1996).

A combination of the use of antibodies raised against both DNA and histone proteins was the basis of a commercial technique marketed as a 'Cell death ELISA' (Figure 10.3). During the initial stages of apoptosis, the plasma membrane becomes relatively impermeable (Piacentini *et al.,* 1994) but the nuclear membrane remains unchanged; as the nuclear DNA breaks down, these fragments can be detected in the cytoplasm (Duke and Cohen, 1986). Recently, Ayala *et al.* (1998) used the technique to study the onset of hepatocellular injury induced by sepsis in mice. The disadvantages of this and any other ELISA-based technique have been reviewed by Allen *et al.* (1997). The major drawback is the inability to relate data to actual cell numbers and the subsequent inability to compare with previous complementary data.

Another popular technique used for apoptosis is the detection of phosphatidyl serine (PS), a negatively charged phospholipid normally restricted to the inner surface of the plasma membrane bilayer (Tilcock *et al.,* 1984). During apoptosis, PS is externalized, acting as a trigger for phagocytosis (Fadok *et al.,* 1992). This process can be monitored using the phospholipid binding protein, annexin V or anti-PS antibodies. Annexin V can be used in live cells and is useful to study the dynamics and rate of apoptosis in cell culture (Ernst *et al.,* 1998, Gatti *et al.,* 1998).

Caspases constitute a family of proteases that are activated at the execution stage of apoptosis (see Chapter 1) by cleavage from a pro-form. Activation of the caspase proteolytic cascade results in apoptotic cell death (see Chapter 2). The activation of caspases during apoptosis together with the discovery of several caspase substrates has led to the development of caspase assays as biomarkers for apoptosis (reviewed in Cohen, 1997, Villa

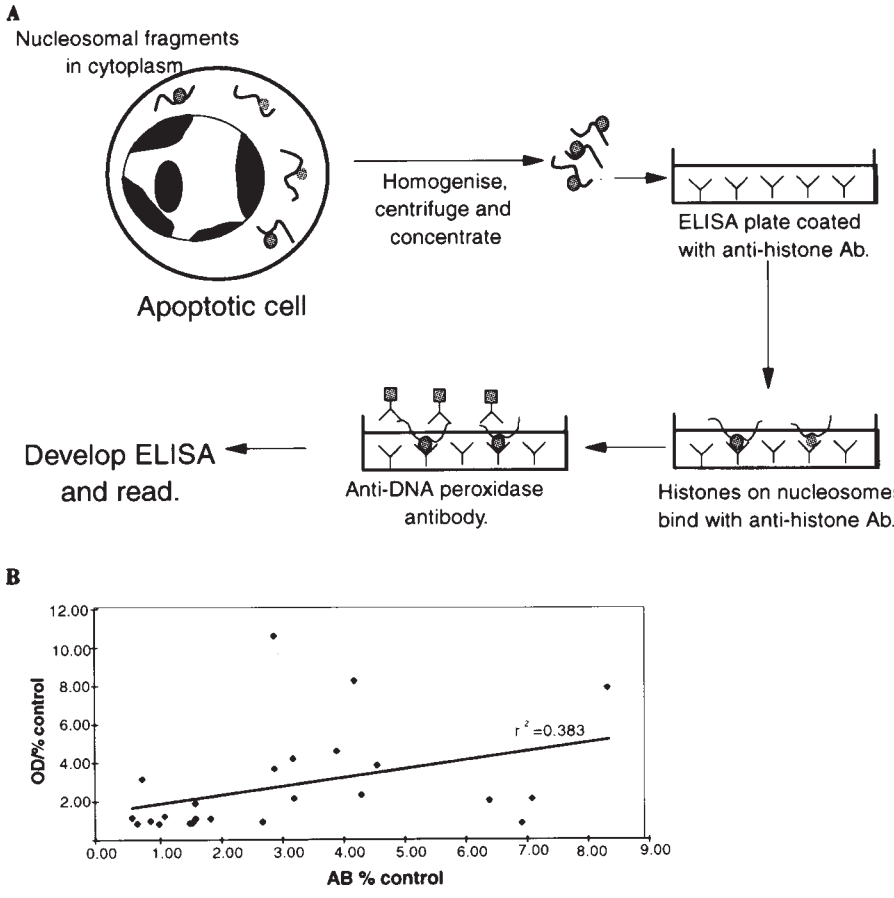

Figure 10.3 (A) Diagramatic representation of the cell death ELISA technology. Anti-histone antibody is adsorbed onto the wall of the titre plate then the nucleosomes contained in the sample bind, via their histone proteins, to the immobilized anti-histone antibodies. A peroxidase-conjugated anti-DNA antibody reacts with the DNA in the bound sample and the amount of antibody bound is directly proportional to the amount of cytoplasmic mono- and oligonucleosomes present in the original tissue (Allen *et al.,* 1997). (B) A scatter diagram of the apoptotic index derived from the cell death ELISA (shown as optical density, OD) compared with the apoptotic index derived from histological sections of TUNEL-stained liver sections (AB% control).

et al., 1997, McCarthy and Evan, 1998). These assays fall roughly into two categories, those that monitor the cleavage of caspases themselves by western blotting and those that measure substrate cleavage either by western blotting or colourimetric/fluorogenic assays. Anti-caspase antibodies are now widely available commercially and can be used to monitor the proteolytic cleavage of key members of the caspase family such as caspase 3, 8 and 10. These types of study provide great detail on the progression of the caspase cascade with time and have been used to determine the roles played be different family members in different tissues. The disadvantages of this approach are antibody specificity

and complexity. For most applications, assays based on the cleavage of caspase substrates are more flexible and practical. Kits that assay cleavage by western blot of the natural caspase-3 substrate, poly (ADP-ribose) polymerase (PARP; see Chapter 2) are now available. In addition, there is a wide range of artificial peptide substrates based on the known caspase cleavage sites linked to a fluorophore or colouriphore (see Chapter 2). Some peptide substrates are highly specific whereas others are cleaved by several caspase family members. Inhibitors are also available, providing additional demonstration of specificity.

10.3.4 Quantitation

The techniques reviewed above are varied but all are aimed at providing a meaningful measurement of the quantity of cell death under different experimental conditions. Converting the raw numbers obtained into such meaningful results presents varied challenges. For methods that count the proportion of apoptotic and viable cells either in cell culture or *in vivo,* the apoptotic index can be calculated directly (James and Roberts, 1996). For *in vivo* estimations, morphological assessment and manual counting of tissue sections is most common by analogy with the labelling index methodology used to count cells that have undergone DNA synthesis (Foster, 1997). Typical methodologies count 2000 cells and evaluate the apoptotic index as a ratio of apoptotic cells to the total (Roberts *et al.,* 1995, Wheeldon *et al.,* 1995, Goldsworthy *et al.,* 1996). However, this may be insufficient due to low basal rates of apoptosis (8–15 apoptotic cells per 10,000 cells in rat liver). Thus, care must be taken to count sufficient cells for meaningful data to be generated. At Central Toxicology Laboratory (CTL) we have developed a TUNEL technique that combines automated image analysis with manual counting (Figure 10.4A). The total number of cells in each lobe of the liver can be estimated by image analysis of the number of nuclei in each section (taken from a mean of 5 random fields) and an estimation of the section area. The selection of 5 fields is based upon the minimum number required to give acceptable standard deviations of within 10% of the mean value. Following this automated assessment of total cell numbers, the total number of apoptotic cells is counted manually by scanning the section systematically. An example of the effect of four chemicals that cause liver growth in rodents on the apoptotic rate present within the liver is given in Figure 10.4B. Despite very low levels of apoptosis in untreated liver, a statistically significant inhibition in the apoptotic index could be detected.

Data obtained from flow cytometry lends itself to quantitation since the apoptotic index is readily derived from the flow profile (Gorman *et al.,* 1997, Al-Rubeai, 1998). For cell suspensions or in systems using lymphocytes, for example, the preparation for flow cytometry is relatively simple and the readout readily understandable (Fraker *et al.,* 1995, Sherwood and Schimke, 1995). Despite this, there are reports where only a limited correlation was seen between estimates of apoptosis by flow cytometry and morphological discrimination (Bryson *et al.,* 1994).

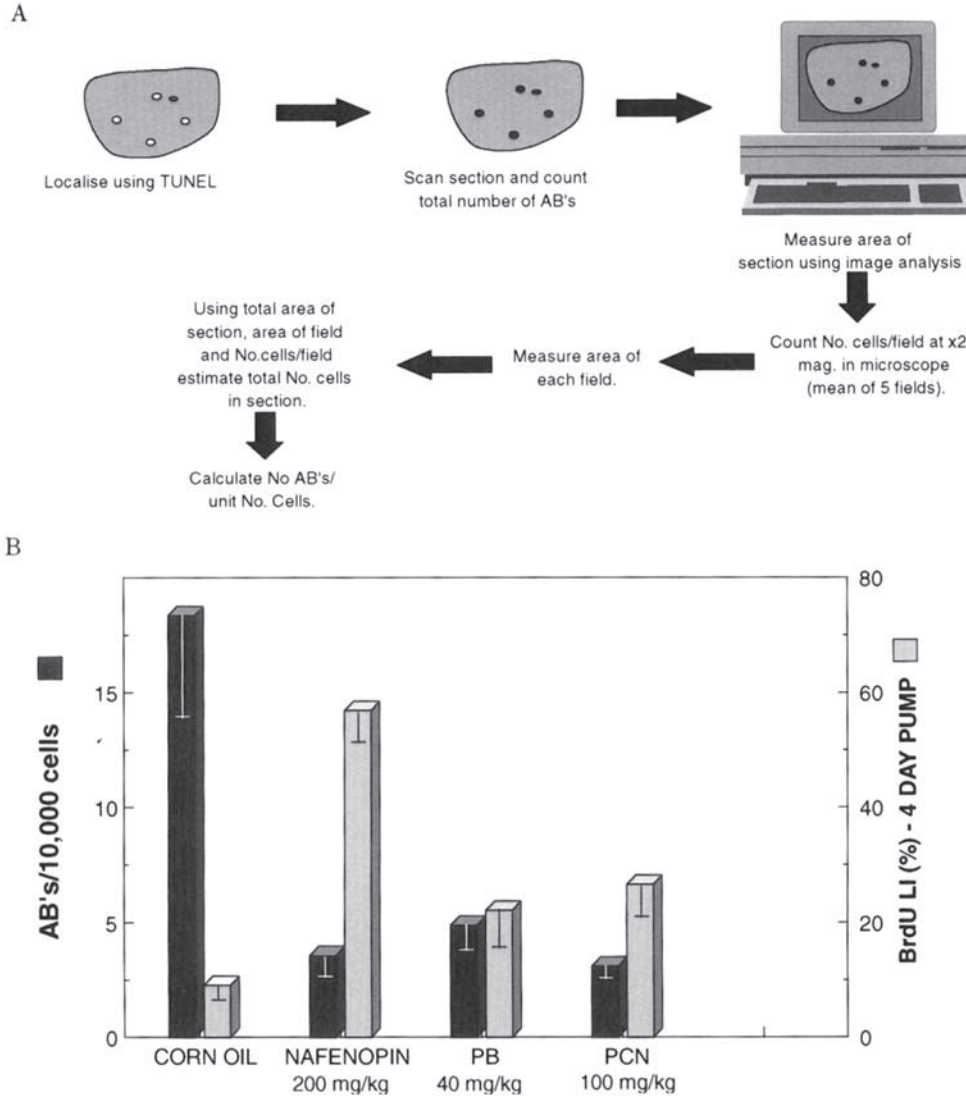

Figure 10.4 (A) Diagram of the methodology used to calculate the apoptotic index in sections of liver stained with the TUNEL technique. (B) The effect of nafenopin, sodium phenobarbitone (PB) and pregnelolone carbonitrile (PCN) on the apoptotic index (AB/10,000 cells) and the S-phase labelling index (BrdU LI) in the livers of rats given the chemicals at the doses shown for 4 days. It can be seen that as the labelling index increases with treatment, the apoptotic index decreases and the livers of the treated rats grow in size.

For ELISA methods, an optical density measurement is provided that requires intepretation from a set of standard curves. As described in section 10.3.3, it may be difficult to refer optical density measurements back to actual cell numbers. Figure 10.3C illustrates a comparison between the apoptotic index derived using the cell death ELISA methodology, described in section 10.3.3, with that derived from a TUNEL procedure.

TABLE 10.3
Comparison of the available techniques for estimating apoptotic index

Technique	Reference
Morphology	
1. Light microscopy	Bursch *et al.*, 1986
2. Electron microscopy	
DNA fragmentation	
1. Light microscopy	Wheeldon *et al.*, 1995
2. Flow cytometry	Gold *et al.*, 1993
Antibody techniques	
1. Light microscopy	Pyrah *et al.*, 1998
2. 'Cell death' ELISA	Ayala *et al.*, 1998
3. Flow cytometry	Dive *et al.*, 1992

10.4 SUMMARY

There are many varied methodologies for evaluating changes in the apoptotic index, each with their own merits (Table 10.3). These will continue to be developed to increase the ease and accuracy with which the relatively rare events of apoptosis can be monitored. Modifications in preparative procedures and increased specificity of the applied techniques, together with the discovery of new, more specific markers promises to make the field ever more productive in the future.

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