

Apoptosis, Cell Signaling, and Human Diseases

Molecular Mechanisms

Volume 1

Edited by

Rakesh Srivastava



HUMANA PRESS

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HUMANA PRESS  TOTOWA, NEW JERSEY

© 2007 Humana Press Inc.
999 Riverview Drive, Suite 208
Totowa, New Jersey 07512

www.humanapress.com

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ANSI Z39.48-1984 (American Standards Institute)

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Cover illustration: Figure 3 of Chapter 4, Volume 2, "Cyclin-Dependent Kinase 5: *A Target for Neuroprotection?*" by Frank Gillardon.

Cover design by Patricia F. Cleary

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Printed in the United States of America. 10 9 8 7 6 5 4 3 2 1

e-ISBN: 1-59745-200-1

Library of Congress Cataloging in Publication Data

ISBN-13: 978-1-58829-677-1
ISBN-10: 1-58829-677-6 (alk. paper)

Preface

The aim of *Apoptosis, Cell Signaling, and Human Diseases: Molecular Mechanisms* is to present recent developments in cell survival and apoptotic pathways and their involvement in human diseases, such as cancers and neurodegenerative disorders. This requires an integration of knowledge from several fields of research, including pathology, genetics, virology, cell biology, medicine, immunology, and molecular biology. This edition of the book examines the impact of molecular biology on disease mechanisms. With recent advances in technology such as microarray and proteomics, new biomarkers and molecular targets have been identified. These potential targets will be very useful for the development of novel and more effective drugs for the treatment of human diseases. The challenge now is not only to understand disease mechanisms but also to apply this knowledge to find therapies that are more effective.

Cellular processes play major roles in cell survival and apoptosis. These events are essential for tissue homeostasis and the maintenance of proper growth and development of multicellular organisms. Imbalance in survival and apoptotic pathways may lead to several diseases. Therefore, understanding the molecular mechanisms of cell survival and apoptotic pathways is essential for the treatment and prevention of human diseases. The main focus of *Apoptosis, Cell Signaling, and Human Diseases: Molecular Mechanisms* is to discuss the recent development in cell signaling events, growth, metastasis, and angiogenesis, mechanisms of drug resistance, and targeted therapy for human diseases. Volume 1 contains 15 chapters divided into two sections: “Malignant Transformation and Metastasis” and “Molecular Basis of Disease Therapy”; Volume 2 contains 18 chapters, also divided into two sections: “Kinases and Phosphatases” and “Molecular Basis of Cell Death.” Scientists well known in their fields have contributed to this book.

In part I, the pathophysiological processes including the mechanisms by which normal cells are transformed to malignant cells, regulation of cell growth, differentiation and apoptosis by oncogenes and tumor suppressor genes, consequences of DNA damage and the ability of cells to repair damaged DNA in response to stress stimuli, molecular events involved in metastasis and angiogenesis, and roles of transcription factors and cytokines in cell survival and apoptosis, are discussed. The recent development in technology has allowed us to identify new diseases before the appearance of the symptoms. The delay in identification of the disease may be fatal to human life. The incorporation of concepts of engineering to the principles of biology has further revolutionized the field of medicine. Nanotechnology, bioinformatics, microarray and proteomics are powerful tools that are being used in drug discovery and development, and treatment of human diseases.

In part II, biological significance of the kinases, and the cell signaling events that control cell survival and apoptosis are discussed. The identification of over 500 protein kinases encoded by the human genome sequence offers one measure of the importance of protein kinase networks in cell biology. Phosphorylation and dephosphorylation of protein kinases such as protein kinase A (PKA), protein kinase C (PKC),

cyclin-dependent kinase (CDK), phosphatidylinositol 3-kinase (PI3K), Akt, and MAP kinase (MAPK) are important for regulating cell cycle, survival, and apoptosis. High-throughput technologies for inactivating genes are producing an inspiring amount of data on the cellular and organismal effects of reducing the levels of individual protein kinases. Despite these technical advances, our understanding of kinase networks remains imprecise. Major challenges include correctly assigning kinases to particular networks, understanding how they are regulated, and identifying the relevant *in vivo* substrates. Genetic methods provide a way of addressing these questions, but their application requires understanding the mutations and how they affect protein-protein interactions.

Apoptosis is a genetically controlled process that plays important roles in embryogenesis, metamorphosis, cellular homeostasis, and as a defensive mechanism to remove infected, damaged, or mutated cells. Molecules involved in cell death pathways are potential therapeutic targets in immunologic, neurologic, cancer, infectious, and inflammatory diseases. Although a number of stimuli triggers apoptosis, it is mainly mediated through at least three major pathways that are regulated by (i) the death receptors, (ii) the endoplasmic reticulum (ER), and (iii) the mitochondria. Under certain conditions, these pathways may cross talk to enhance apoptosis. Death receptor pathways are involved in immune-mediated neutralization of activated or autoreactive lymphocytes, virus-infected cells, and tumor cells. Consequently, dysregulation of the death receptor pathway has been implicated in the development of autoimmune diseases, immunodeficiency, and cancer. Increasing evidence indicates that the mitochondrial and ER pathways of apoptosis play a critical role in death receptor-mediated apoptosis. Dysregulation of these pathways may contribute to drug resistance.

A lot of progress has been made in understanding the mechanisms of apoptosis. Mitochondria are critical death regulators of the intrinsic apoptotic pathway in response to DNA damage, growth factor withdrawal, hypoxia, or oncogene deregulation. Activation of the mitochondrial pathway results in disruption of mitochondrial homeostasis, and release of mitochondrial proteins. The release of mitochondrial apoptogenic factors is regulated by the pro- and anti-apoptotic Bcl-2 family proteins, which either induce or prevent the permeabilization of the outer mitochondrial membrane. Activation of the death receptor pathway also links the cell-intrinsic pathway through Bid. Mitochondrial membrane permeabilization induces the release of mitochondrial proteins (e.g., cytochrome c, Smac/DIABLO, AIF, Omi/HtrA2, and endonuclease G), which are regulated by proapoptotic and antiapoptotic proteins of Bcl-2 family, and in caspase-dependent and -independent apoptotic pathways. The antiapoptotic members (e.g. Bcl-2 or Bcl-X_L) inhibit the release of mitochondrial apoptogenic factors whereas the proapoptotic members (e.g. Bax, and Bak) trigger the release.

Recent studies suggest that, in addition to mitochondria and death receptors, other organelles, including the endoplasmic reticulum (ER), Golgi bodies, and lysosomes, are also major points of integration of proapoptotic signaling and damage sensing. Each organelle possesses sensors that detect specific alterations, locally activate signal transduction pathways, and emit signals that ensure inter-organellar cross-talk. The genomic responses in intracellular organelles, after DNA damage, are controlled and amplified in the cross-signaling via mitochondria; such signals induce apoptosis, autophagy, and other cell death pathways.

Chromatin remodeling agents modulate gene expression in tumor cells. Acetylation and deacetylation are catalyzed by specific enzyme families, histone acetyltransferases (HATs) and deacetylases (HDACs), respectively. Since aberrant acetylation of histone and nonhistone proteins has been linked to malignant diseases, HDAC inhibitors bear great potential as new drugs due to their ability to modulate transcription, induce differentiation and apoptosis, and inhibit angiogenesis. The pre-clinical data on HDAC inhibitors are very promising, and several HDAC inhibitors are currently under clinical trials for the treatment of cancers.

Apoptosis, Cell Signaling, and Human Diseases: Molecular Mechanisms will be valuable to graduate students, postdoctoral and medical fellows, and scientists with a working knowledge of biology and pathology who desire to learn about the molecular mechanisms of human diseases and therapy. I hope that individuals of diverse backgrounds will find these volumes very useful.

Rakesh Srivastava, PhD

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MALIGNANT TRANSFORMATION AND METASTASIS

***BCR-ABL* and Human Cancer**

María Pérez-Caro and Isidro Sánchez-García

Summary

The *BCR-ABL* oncogene was the first chromosomal abnormality shown to be associated with a specific human malignancy, the chronic myelogenous leukemia (CML), resulting from a reciprocal t(9;22) translocation characterized by the formation of a shortened chromosome, named Philadelphia chromosome (Ph), in which the tyrosine kinase of c-ABL is constitutively activated. This chromosomal translocation generates *BCR-ABL* fusion genes which can be translated in three different oncoproteins, p210, p190, and p230, associated with three different pathologies in humans, CML, acute lymphoblastic leukemia (ALL), and chronic neutrophilic leukemia, respectively. The molecular mechanisms and downstream pathways of *BCR-ABL* are poorly understood mainly as a result of the lack of a good in vivo model that mimics the human disease. Nevertheless, additional in vitro and in vivo models have led to the design of several novel therapeutic approaches. That is the case of Imatinib mesylate (Gleevec, STI571; Novartis Pharma AG), a drug targeting the tyrosine kinase activity of *BCR-ABL* and an effective therapy for chronic phase CML but not advanced stages of CML and patients with Ph⁺ ALL. The resistance mutations in the kinase domain of *BCR-ABL* together with the stem cell origin of the chromosomal translocation and the inability of the imatinib to inhibit the *BCR-ABL* activity in these cells have revealed the limitations of Gleevec, providing new lessons for the development of alternative therapies in oncology.

Key Words: *BCR-ABL*; chronic myelogenous leukemia (CML); cancer stem cells; stem cells; mouse models; human cancer; disease progression; tyrosine kinase inhibition; imatinib mesylate.

1. Introduction: The Philadelphia Chromosome

The Philadelphia (Ph) chromosome was the first specific cytogenetic lesion associated with a human malignant disease, namely chronic myeloid leukemia (CML) (1). It took 13 yr before it was appreciated that the Ph chromosome is the result of a t(9;22) reciprocal chromosomal translocation and another decade before this translocation was shown to involve the *ABL* proto-oncogene, normally in chromosome 9, and a previously unknown gene, later termed *BCR* for breakpoint cluster region, on chromosome 22 (2). The regulated activity tyrosine kinase of ABL was then defined as the pathogenic principle of a defined biphasic disease of the hematopoietic system; the initial chronic phase, characterized by an excessive production of myeloid cells that retain a normal differentiation program, and an acute phase termed “blast crisis” which resembles acute leukemia (3). In order to understand the mechanisms of the Ph chromosome and malignancy, it is necessary to know the biology and functions of the translocated genes *ABL* and *BCR*.

The proto-oncogene c-*ABL* belongs to the family of the nonreceptor tyrosine kinases (TKs) and was originally identified for its homology with v-*ABL* (Abelson murine

leukemia virus) and ability to induce an acute neoplastic transformation in mice (4). The mammalian *c-ABL* gene is ubiquitously expressed. It encodes two 145 kDa isoforms arising from alternative splicing of two distinct first exons (1a/I and 1b/IV in the human and mouse respectively) (5). The *c-ABL* proto-oncoprotein is normally distributed both in the nucleus (where it is bound to chromatin) and in the cytoplasm (where it co-localizes with F-actin), but it is principally found in the nucleus (6). The pattern of expression and the endocellular location of the *c-ABL* protein suggest that this molecule plays an important role in cellular biology and also exerts multiple functions in various cell compartments; however, the exact role of *c-ABL* still needs further clarification (7). To realize the possible functions of *c-ABL* at both the cytoplasmic and nuclear level, it is also important to consider the different domains present in the *c-ABL* protein.

As in nonreceptor TKs, the *c-ABL* protein possesses three Src homology (SH) regions—1, 2, and 3—at the N-terminal extremity that mediate catalytic (SH1), phospho-tyrosine, and proline-rich sequence binding proteins (SH2–SH3), respectively (8). The C-terminal extremity of this protein contains a domain of interaction with F-actin that suggests an important role of *c-ABL* in the mechanisms which regulate the variations of the cellular morphology and the intercellular adhesion (9). Indeed, several diverse functions have been attributed to *c-ABL*. The normal *ABL* protein is involved in the regulation of the cell cycle (10), in the cellular response to genotoxic stress (11), and in the transmission of information about cellular environment through integrin signaling; but overall, it appears that this protein serves as a cellular module that integrates signals from various extracellular and intracellular sources and that influences decisions with regard to cell cycle and apoptosis (12).

In the event of translocation, *c-ABL* from chromosome 9 associates with a portion of a gene on chromosome 22 termed *BCR*. Instead of the small portion of the *BCR* gene overlapping the breakpoint region, the entire gene is relatively large and consists of about 25 exons, including two putative alternative first (e1') and second (e2') exons (13). At the end, a 160-kDa protein is formed and, as with *c-ABL*, is ubiquitously expressed in the cell. The first N-terminal exon encodes for a region with serine-threonine kinase activity. A coiled-coil domain at the N-terminus of *BCR* allows dimer formation in vivo (14). The center of the molecule contains a region with *dbl*-like (from the *DBL* proto-oncogene) and pleckstrin homology (PH) domains that stimulate the exchange of guanidine triphosphate (GTP) for guanidine diphosphate (GDP) on Rho guanidine exchange factors. The C-terminus encodes a GTPase activating protein for $p21^{rac}$ (15), an essential activating component of the neutrophil respiratory burst. In addition, *BCR* can be phosphorylated on several tyrosine residues, especially tyrosine 177 (which binds Grb-2), involved in the activation of the Ras pathway (16). Although these and other data argue for a role of *BCR* in signal transduction, their true biological relevance remains to be determined.

1.1. *BCR-ABL* Chromosomal Translocation: $p210^{BCR-ABL}$ and Chronic Myeloid Leukemia

Although the presence of a Ph chromosome translocation always parallels the presence of a *BCR-ABL* rearrangement, there is variability at the molecular level concerning the type of rearrangement between *BCR* and *ABL*. As a consequence, *BCR-ABL* hybrid genes can generate different types of fusion transcripts and proteins showing a

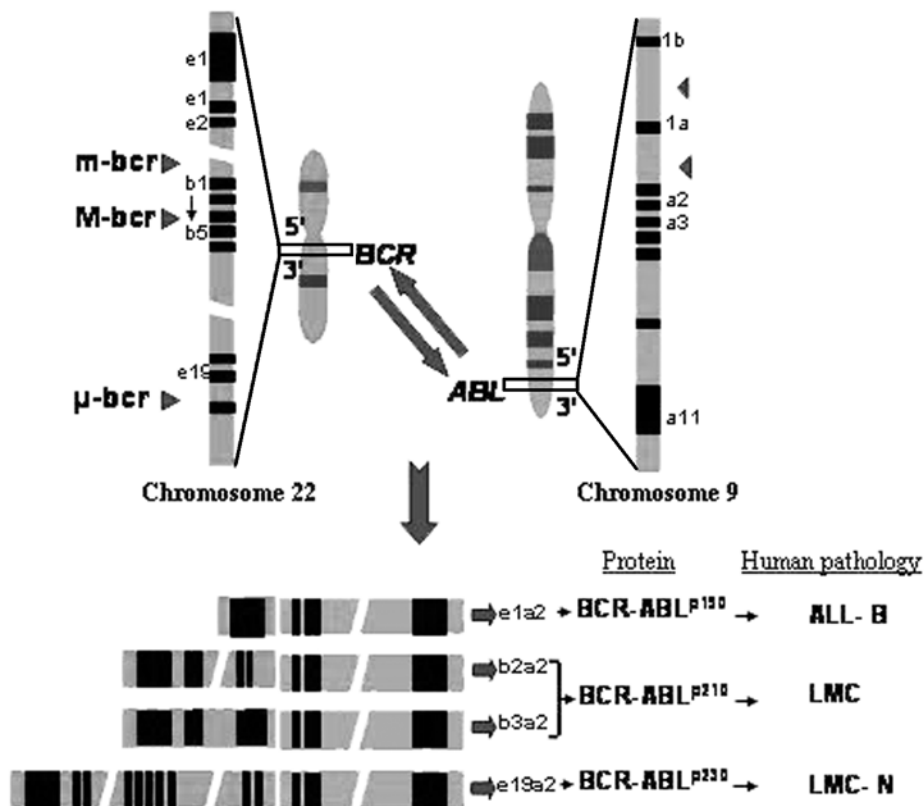


Fig. 1. The chromosomal translocation $t(9;22)(q34;q11)$. The Ph chromosome is a shortened chromosome 22 that results from the translocation of 3' *ABL* segments on chromosome 9 to 5' *BCR* segments on chromosome 22. Breakpoints (arrowheads) on the *ABL* gene are located 5' (toward the centromere) of exon a2 in most cases. Various breakpoint locations have been identified along the *BCR* gene on chromosome 22. Depending on which breakpoints are involved, different-sized segments from *BCR* are fused with the 3'-sequences of the *ABL* gene. This results in chimeric protein products (p190, p210, and p230) with variable molecular weights and presumably variable function. The abbreviation m-bcr denotes minor breakpoint cluster region, M-bcr major breakpoint cluster region, and μ -bcr a third breakpoint location in the *BCR* gene that is downstream from the M-bcr region between exons e19 and e20.

preferential but not exclusive association with different leukemia phenotypes (17,18). The breakpoints within the *ABL* gene at 9q34 can occur anywhere over a large (>300 Kb) area at its 5'-end, either upstream of the first alternative exon 1b, downstream of the alternative exon 1a, or, more frequently, between both. Interestingly, exon 1, even if retained in the genomic fusion, is never part of the chimeric mRNA (17). All *BCR-ABL* fusion genes contain a variable 5' portion derived from *BCR* sequences and a 3' portion almost invariably of the *ABL* gene sequence (see Fig.1).

In CML, the breakpoints on chromosome 22 are restricted to a central region of the *BCR* gene called major breakpoint cluster region (M-BCR). This region contains five exons corresponding to *BCR* exons 10 to 14, but originally numbered from 1 to 5. Generally, the break occurs within introns located between exons b2 and b3 or b3 and

b4 with the *ABL* exon a2, forming the fusion gene b2a2 or b3a2, respectively. The translocation product of this *BCR-ABL* fusion gene is a novel chimeric 210 kDa protein termed p210^{*BCR-ABL*}. The presence of this characteristic Ph chromosome p210^{*BCR-ABL*} is the hallmark cytogenetic event in about 90% of the cases of CML.

The natural history of CML ends with an acute leukemia phase usually fatal in a few months. CML arises from the clonal expansion of a single pluripotent hematopoietic stem cell. It follows a biphasic course with an initial phase of myeloid hyperplasia in which myeloid and other hematopoietic cell lineages are involved in the process of clonal proliferation and differentiation (18). Nevertheless, the initial expansion results not only from an increased rate of proliferation but also from the occurrence of additional cell divisions during myeloid maturation and the ability of CML clones to reach higher cell densities than normal with enhanced cell survival (19). The *BCR-ABL* oncoprotein has constitutively expressed tyrosine kinase activity as a result of oligomerization of the coiled coil region of p210^{*BCR-ABL*} and deletion of the inhibitory SH3 domain of *ABL*. This results in phosphorylation of p210^{*BCR-ABL*} itself on the Y-177 tyrosine residue and leads to recruitment of GRB2 (20), a small adapter molecule that can activate the RAS pathway, and in the phosphorylation of JAK2 and STAT1/STAT5 (21,22), whose activation contribute to growth factor independence. Furthermore, p210^{*BCR-ABL*} activates the PI(3)/Akt pathway (23,24), increases expression of Bcl-2 (25), and phosphorylates STAT5 (26) leading to the increased resistance of CML progenitors to apoptosis. Finally, p210^{*BCR-ABL*} is localized almost exclusively in the cytosol because of the loss of a nuclear localizing signal and thus has increased binding to actin as compared with c-*ABL* (27,28). This results in phosphorylation of a number of neighboring cytoskeletal proteins that contribute to the aberrant adhesion receptor function and may explain the premature circulation of progenitors and precursors of the blood (29).

Therefore, the features of chronic phase CML, expansion and premature circulation of the malignant myeloid population, can be explained by activation of mutagenic pathways, antiapoptotic pathways, and abnormal cytoskeletal function. The same characteristics—increased mutagenicity and decreased susceptibility to apoptosis—may also be responsible for disease progression (30).

After variable periods, CML may enter an advanced blast phase characterized by the appearance of poorly differentiated Ph⁺ myeloid or lymphoid blast cells in the peripheral blood. *BCR-ABL* activity promotes the accumulation of molecular and chromosomal alterations directly or indirectly responsible for the reduced apoptosis susceptibility and the enhanced proliferative potential and differentiation arrest of CML blast-crisis cells. This phenotype is normally accompanied by nonrandom clonal chromosomal defects, the duplication of the Ph chromosome being the most frequent defect that increases the levels of both *BCR-ABL* mRNA and the protein associated with disease progression, trisomy 8, trisomy 19, and an isochromosome 17q replacing the normal 17 (31). The abnormalities in other proto-oncogenes or tumor suppressor genes during the blast crisis include point mutations in the coding sequences of *RAS* (32), *p53* (33), gene amplification of *MYC* that occurs as a result of increased transcription or trisomy 8 (34), or rearrangements of *Rb* (35) and *p16* (36). The suppressor gene *16^{INK4a}*, located on chromosome 9, is an inhibitor of cyclin D that phosphorylates Rb, thus preventing cell cycle arrest in G1 (37). Sequential studies of CML patients demonstrate homozygous deletions acquired in association with the progression to lymphoid blast

crisis in approx 50% of cases (38). Moreover, epigenetic alterations, such as the methylation of several promoters (including the proximal promoter of *c-ABL* oncogene), appear to be specifically and consistently associated with the progression of CML (39,40). Silencing of the *c-ABL* promoter by methylation will further decrease nuclear *c-ABL* levels, thereby further decreasing apoptosis and, perhaps, enhancing genomic instability, features that are all related to disease progression.

BCR-ABL utilizes a number of alternative pathways or multiple signaling pathways to transform cells and protect them from cell death. Although the relative importance of these pathways in the natural history of the disease remains to be elucidated, the identification and cloning of molecules that are part of the signaling cascade in leukemic cells creates new possibilities for the development of more effective therapeutic compounds. One of these molecules was recently identified because of its potentially important role in cancer dissemination is *SLUG* (41). *SLUG* (*SNAI2*), a member of the snail family of zinc-finger transcription factors, was first identified because of its critical role for the normal development of neural crest-derived cells and development of the mesoderm in chick embryos (42). Much of the knowledge regarding the function of this gene in humans and mice was derived from analysis of loss-of-function mutations (43–46); however, little was known about how the aberrant expression of *SLUG* contributed to malignancy. The generation of a Slug-overexpressing mouse model (41) aided in the understanding of *SLUG*'s relevance to human cancer development (41,46–48). Slug-expressing mice developed mesenchymal cancers, mainly leukemias, facilitated cell migration, and promoted survival of these migrating cells (49). In addition, *BCR-ABL* oncogenes induced *SLUG* expression, showing that the leukemogenic capacity of the p190^{*BCR-ABL*} oncogene is dependent on the presence of *SLUG* (46). All these results were consistent with a model in which tumor cells harboring the *BCR-ABL* fusion protein constitutively express *SLUG*, promoting both aberrant survival of tumor cells and migration of defective target cells (see Fig. 2). *SLUG* expression might, therefore, be defined as a common pathway of cell invasion for other leukemias and sarcomas, representing an important mechanism of tumor invasion as well as an attractive target for therapeutic modulation of invasiveness in the treatment of human cancer (41).

1.2. Other Forms of BCR-ABL Preferentially Associated With Specific Human Leukemia

As it was introduced previously, the presence of a Ph chromosome translocation always parallels the presence of a *BCR-ABL* rearrangement whose variability at the molecular level depends only on the type of rearrangement between both implicated genes. All *BCR-ABL* fusion genes contain a 5' portion derived from *BCR* sequences and a 3' portion that, with very few exceptions, includes the entire *ABL* gene sequence with the exclusion of the first two alternative exons, 1a and 1b, which never form part of the chimeric mRNA. Instead of the p210^{*BCR-ABL*} fusion transcript resulting from the alternative splicing between the M-*BCR* and the *ABL* exon 2a; there are two other breakpoint cluster regions located in *BCR* that are characterized as minor-*BCR* (m-*BCR*) and micro-*BCR* (μ -*BCR*). The m-*BCR* is located upstream of M-*BCR* in the long 54.4 kb intron between the two alternative exons e2' and e2. The translation product of this fusion gene, which is transcribed into an e1a2 7.5 kb mRNA, is a 190-kDa protein, designated p190^{*BCR-ABL*}, and is associated with Ph-positive ALL in 70% of

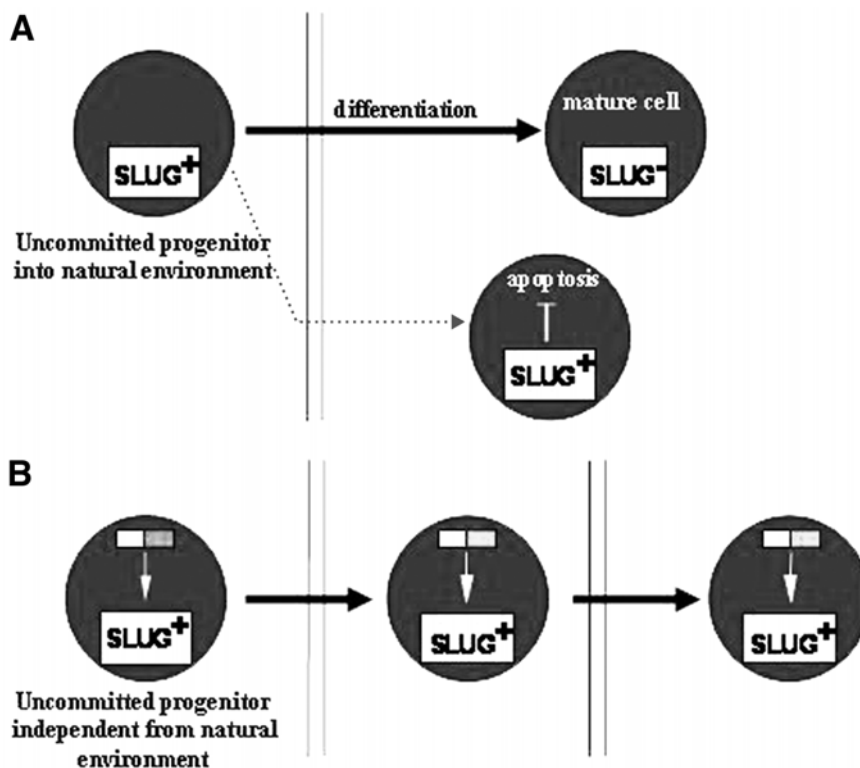


Fig. 2. Model of the role of Slug in BCR-ABL-dependent cancer. **(A)** In the hematopoietic system, normal uncommitted progenitor cells differentiate to mature cells. During this transition, the expression of Slug is downregulated (179,180). These normal uncommitted progenitor cells are responsive to environmental cues, which regulate the number of mature cells produced and limit the self-renewal of immature cells. When in physiological situations, these normal uncommitted progenitor cells migrate, Slug would promote survival to allow them to carry out their function. If this is not achieved in a specific period of time, they would undergo apoptosis as they have been deprived of required external signals (41). **(B)** In cancer development, the differentiation capacity of the target cell is blocked, but inhibition of differentiation is not sufficient for transformation because survival and proliferation of target cells would be restricted to a particular microenvironment. Thus, other genetic changes that allow cells to grow outside their normal environment in addition to mutations that block differentiation must exist. *BCR-ABL* fusion oncogenes created as a result of chromosomal abnormalities associated with leukemias block differentiation and have the capacity to activate target genes such as Slug, which promotes survival (with independence of the required external signals) and migration of the defective target cells into different environments (41).

cases (50). Both in vivo and in vitro studies suggest that P190 is characterized by a higher transforming activity than P210, and this could explain why P190 is preferentially associated with an acute leukemia phenotype. However, the relationship between the BCR-ABL fusion protein and the leukemia phenotype is an intriguing question. Although the P190 fusion protein is almost exclusively associated with an acute leukemia phenotype, mainly lymphoid, there are also rare chronic-phase CML cases which exclusively express P190 instead of P210 (18). These P190 CMLs frequently

appear to be associated with the chronic myelomonocytic leukemia like (CMML) phenotype (51). It appears that the presence of p190^{BCR-ABL} in a committed early myeloid cell could result in a myeloproliferative defect that includes the monocytic lineage, whereas p210^{BCR-ABL} in the same type of progenitor restricts the excessive proliferation to the granulocytic pathway. Finally, a longer type of *BCR-ABL* transcript in which the breakpoint takes place at the very 3' end of the *BCR* gene (m-*BCR*) and joins *BCR* exon 19 with *ABL* exon 2 (e19a2) was originally described 15 yr ago (52); this transcript includes the same portion of the *ABL* sequence as the other more common types but contains almost all of the *BCR* coding sequences, resulting in a fusion chimeric protein of 230 kDa (p230^{BCR-ABL}) being present in a subset of patients with Ph+ chronic neutrophilic leukemia (CNL). Thus, p230^{BCR-ABL} contains additional sequences that are not found in the p190 or p210 variants, specifically the calcium phospholipid binding (CalB) domain and two-thirds of the domain associated with the GTPase activating activity for p21rac (GAP). CNL is a rare disorder marked by a sustained mature neutrophilic expansion typically more indolent as compared with classical CML; progression to blast crisis is uncommon (53).

Based on the observation that the *ABL* part in the chimeric protein is almost invariably constant whereas the *BCR* portion varies greatly, one may deduce that *ABL* is likely to carry the transforming principle and the different sizes of the *BCR* sequence may dictate the phenotype of the disease.

1.3. *TEL (ETV6)-ABL and Rare Human Leukemias*

Instead of the classical rearrangement between *BCR* and *ABL* genes, several patients with clinical features of CML were found to have a different fusion of the *TEL (ETV6)* gene with *ABL* expressing a chimeric TEL-ABL protein that contains the same portion of the *ABL* tyrosine kinase fused to TEL rather than to *BCR*. However, this fusion in human acute or chronic leukemia appears to be a rare event (54,55).

The *TEL* gene (also known as *ETS*-variant gene 6 [*ETV6*]), is located on chromosome 12q13 and was originally identified at the breakpoint of a (5;12) translocation in a patient with CMML, where it was fused to the platelet-derived growth factor β (*PDGF- β R*) receptor gene (56). *TEL* encodes a ubiquitously expressed 452 amino acid protein with two regions of homology to the Ets family of transcription factors: the pointed (PNT) homology domain and the DNA binding domain. Subsequently, *TEL* was recognized to be rearranged in many different chromosomal translocations in human leukemia such as t(12;21) in pre-B ALL in which *TEL* is fused to *AML1* gene (57).

The *TEL-ABL* fusion oncogenes produce two different TEL-ABL proteins; patients with B-lymphoblastic leukemia (B-ALL) and atypical CML; the first 4 exons of *TEL* are fused to *ABL* exon 2; the other patients had *TEL* exons 1 through 5 fused to *ABL* exon 2. The result is two fusion proteins with increased tyrosine kinase activity that are localized to the cytoplasm and F-actin cytoskeleton (58,59), similar to BCR-ABL.

In *TEL*, the PNT domain mediates oligomerization (58) and is required for activation of the TEL-PDGF- β R (60), TEL-JAK2 (61), and TEL-ABL (58) fusion tyrosine kinases. The fact that BCR contains a coiled-coil oligomerization domain also requires for activation of BCR-ABL kinase activity and transformation (62) has led to the suggestion that oligomerization mediated for these partners of *ABL* may be the critical event in the pathogenesis of these leukemias.

1.4. BCR-ABL in Healthy Individuals

With rare and questionable exceptions, all patients with bona fide CML have a *BCR-ABL* rearrangement in their malignant cell clone. In the same way, the finding of a *BCR-ABL* fusion gene in hematopoietic cells is a sign of impending or overt CML (or Ph⁺ ALL, and AML). Nevertheless, there is some indirect evidence that the formation of the *BCR-ABL* hybrid gene may be detected at low frequency in the blood of many healthy individuals (63,64). These data have important biological implications because they suggest that in order to successfully produce a leukemic phenotype it is necessary that the fusion gene structure allow for the production of a functional protein with direct or indirect oncogenic properties and that the chromosomal translocation occur in a relatively early precursor cell with self renewal capacity. In other words, only the combination of a correct fusion gene in the correct primitive hematopoietic progenitor has the potential selective advantage and can successfully become an expanding clone (64–66).

It is therefore likely that the *BCR-ABL* genes detected in the circulating leukocytes from healthy individuals (67) do not reflect incipient leukemia, because they were generated by relatively mature, harmless progenitors from which the derived clones are eventually lost through normal cell differentiation and death.

2. The In Vitro BCR-ABL Induced Transformation is Influenced by the Cellular Context

Despite the large body of data accumulated in recent years, the molecular pathways by which BCR-ABL proteins induce transformation remain, in part, obscure. To elucidate the molecular pathogenesis of CML and to identify targets for therapeutic intervention for CML, the roles and relative importance of the domains of *BCR-ABL*, of the *BCR-ABL*-activated signaling pathways and of the micro-environment of BCR-ABL-targeted cells in neoplastic transformation by *BCR-ABL* must be examined in biological model systems.

A variety of model systems for *BCR-ABL* transformation with different advantages and disadvantages have been developed and used.

Fibroblast lines have been used extensively in CML research because of their easy manipulation. Fibroblast transformation, anchor-independent growth in soft agar, is the standard in vitro test for tumorigenicity (68). Conversely, it became clear that the introduction of *BCR-ABL* into fibroblasts has diverse effects depending of the type of fibroblast used. p210^{*BCR-ABL*}, for example, is able to transform Rat-1 fibroblasts (69) but not NIH3T3 (70). Indeed, NIH3T3 fibroblasts are not transformed by either of the two *BCR-ABL* oncogenes or by *v-ABL* itself (69). However, it has been shown that p210^{*BCR-ABL*} and *v-ABL* convert the factor-dependent Ba/F3 cell line into factor-independent and make it tumorigenic (71), preventing apoptotic death by inducing a *BCL-2* expression pathway (72). These different results were obtained from studies in fibroblasts and must consequently be interpreted carefully.

Hematopoietic cell lines as in vitro CML models, including cell lines with myeloid differentiation such as the well-known K562, allow for fairly good study of the blast-crisis phase but are insufficient as models of chronic-phase CML. Although all these lines are derived from blast crisis, they contain genetic lesions in addition to *BCR-ABL* and their immortalization is not successful because of their limited life span (73), in contrast with their Ph[–] counterparts.

Transformation of factor-dependent cell lines to growth factor independence is an important feature of BCR-ABL and other oncoproteins that contain an activity tyrosine kinase (58,74). Murine cell lines such as Ba/F3 and 32D and human cell lines such as MO7 have been used to study the effects of *BCR-ABL*. A particular advantage of murine lines is the fact that they are derived from nonmalignant hematopoietic cells; this, unfortunately, does not rule out the development of additional mutations (75) that confer a selective growth advantage.

The advance of using cell lines is the relative ease of obtaining a large number of clonally derived cells for biochemical analysis, genetic manipulation, and biological examination. It is particularly important to use the same cellular context for studies that compare cellular and molecular events affected by various *ABL* oncogenes or their mutants where the oncogenes may have different oncogenic potentials in different blood lineages in vivo. Moreover, because the tyrosine kinase activity of BCR-ABL, as well as other oncoproteins with an intrinsic tyrosine kinase activity, is essential for its oncogenic potential, the factor-dependent hematopoietic cell lines are very useful for searching and testing specific pharmacological inhibitors which should restore the factor-dependence of these cell lines (76–78). However, development of leukemia is a complex process that involves both the effects of *BCR-ABL* within its target cells and the interactions of *BCR-ABL* target cells with the rest of the in vivo environment. Cell lines are limited in representing the physiologically relevant target cells of BCR-ABL in vivo.

To overcome the limitations of established cell lines, primary cells isolated from mice or humans have also been used to study *BCR-ABL* transformation. It has been shown that *BCR-ABL* is able to stimulate cytokine-independent growth of primary bone marrow cells and to stimulate growth of hematopoietic cells differentiated from embryonic stem (ES) cells; adapting an ES cell differentiation system on a macrophage colony stimulating factor (M-CSF)^{-/-} stromal cell line (OP9) preferentially supports differentiation of myeloid and erythroid cells without overproduction of macrophages, favoring hematopoietic development (79) and inducing their differentiation into hemangioblasts capable of forming hematopoietic and endothelial cells.

In these studies, the oncogene expression increased the percentage and absolute number of immature progenitor elements and the normal balance of erythroid to myeloid colonies was reversed to a dominance of myeloid over erythroid (80), reproducing one cardinal feature of the clinical disease. In vitro ES-differentiated progenitors can contribute to long-term, multilineage hematopoiesis in vivo (81), and *BCR-ABL*-expressing ES-derived hematopoietic progenitor clones generate an acute leukemia in vivo (82), suggesting that ES-derived hematopoietic progenitors may be a potential cell population that can be used to study *BCR-ABL* effects in vivo.

Taking all these assays together, there is no doubt that the study of cell lines contributed significantly to the understanding of CML. Although these systems can overcome some disadvantages of established cell lines, they are still limited in representing in vivo biology of the disease.

3. Animal Models

The deficiencies of the in vitro assays and the lack of a functional assay using human cells have driven the search for a murine CML model that accurately recapitulates this

disease. Because CML is a stem cell disease, it is critical to model the disease by targeting the *BCR-ABL* oncogene into multipotential hematopoietic stem and progenitor cells and express it in these cells and their progenies, similar to the way *BCR-ABL* is generated and expressed in CML patients. Instead of engrafting human CML cells in immunodeficient mice, there are three methods that are generally used for targeting oncogenes into mice transgenic, knock-in, and retroviral transduction. All of these approaches, as well as some others, have been used for investigating *BCR-ABL* leukemogenesis.

3.1. Syngeneic Mice

Murine factor-dependent cell lines, such as 32D or BaF3, transduced with the translocation *BCR-ABL* oncogene give rise to an aggressive leukemia when transplanted into mice with the same genetic background; these are known as syngeneic mice. Infection with a p210^{*BCR-ABL*}-expressing retrovirus leads to the outgrowth of clonal cell lines with the phenotypic properties of immature lymphoid cells. Inoculation of these cells into syngeneic mice allows tumor progress to be followed from localized masses at the site of intraperitoneal injection to the spleen and other lymphoid organs (83). After 2 wk, tumors are widely disseminated and simulate a form of acute leukemia more than chronic phase CML. Although it is not a good model for the study of initial chronic phase CML, it constitutes an excellent *in vivo* model to test the efficacy of new drugs *in vivo*.

3.2. Engraftment of Immunodeficient Mice With Human *BCR-ABL* Positive Cells

Cell lines derived from human blast crisis are propagated relatively easily in severe combined immunodeficiency (SCID) mice (84). In addition to the SCID defect in V(D)J recombination, these animals lack functional natural killer (NK) cells. Chronic-phase CML cells and, even more so, cells from accelerated phase or blast crisis readily engraft in these mice, showing a significant correlation between engraftment and disease state.

Early attempts by many groups to establish an *in vivo* transplantation model of chronic phase CML by using SCID mice as recipients of human cells were not successful (85,86). Even blast crisis CML cells, when injected intraperitoneally into SCID mice, under the renal capsule, or into subcutaneously implanted human fetal bone fragments, were found to disseminate poorly to the bone marrow of the mice (86,87). Whereas the two growth-factor independent cell lines K562 and U937 grew aggressively and induced leukemia in these animals, three other myeloid cell lines, which require interleukin 3 or granulocyte-macrophage colony-stimulating factor (GM-CSF) for continuous growth *in vitro*, failed to induce disease.

The discovery that nonobese diabetic NOD/SCID mice have additional defects in NK cell activity as well as defective macrophage and complement function (88) has allowed superior engraftment of normal (89,90) and leukemic (91) human hematopoietic cells, proving that they are better recipients for CML cells. Through comparison with the SCID model, engraftment of NOD/SCID mice with multiple types of normal and leukemic human cells was higher and could be achieved with lower numbers of CD34⁺ cell enriched populations. These experiments provide a foundation for both the future characterization of the phenotype and properties of normal and Ph⁺ cells that have long-term *in vivo* repopulating activity as well as for the development of strategies to selectively manipulate these populations *in vivo*.

3.3. Transduction of Murine Bone Marrow Cells With BCR-ABL Retroviruses

In 1990, several groups reported that a CML-like myeloproliferative syndrome could be induced when p210^{BCR-ABL}-infected marrow was transplanted into syngeneic recipients (92–94). However, in these first bone marrow transplantation experiments, mice that received bone marrow cells transduced with p210^{BCR-ABL} also developed other hematopoietic neoplasms such as B-ALL and macrophage tumors. More importantly, the low efficiency of inducing a myeloproliferative disorder (MPD) combined with poor reproducibility made them useful in studying the biology of *BCR-ABL* in CML. The improvement of BCR-ABL retroviral stocks, with refined culture conditions and more efficient expression of *BCR-ABL* in desired targets cells, has allowed for more accurate reproduction of human CML in mice (95,96). Common features of the disease include increased numbers of peripheral blood cells (PBCs) (with a predominance of granulocytes), splenomegaly, and extramedullary hematopoiesis in liver and pulmonary haemorrhages resulting from extensive granulocyte infiltration in the lung. Additionally, the disease is mostly polyclonal and can be transplanted into secondary recipient mice. The ability of *BCR-ABL* to induce a much more efficient and reproducible CML-like MPD in mice in this model likely resulted from proficient expression of the transgene in the correct cell type, the hematopoietic stem cell and progenitor cell (97,98). Nevertheless, pulmonary hemorrhage, a complication not found in human CML, was a frequent cause of death in these studies, indicating that these novel models may have their own distinct problems even though they have allowed for the identification of targets both within and downstream of BCR-ABL.

3.4. Transgenic Mouse Models

Until quite recently, the design of transgenic mouse models of human CML had been unsuccessful, probably because of the lack of an appropriate promoter for the direct expression of the translocated *BCR-ABL* gene. In human disease, p210^{BCR-ABL} is expressed under the control of the *BCR* promoter. However, *BCR* promoter directed expression of p210^{BCR-ABL} results in embryonic lethality in mice (99); thus, in order to create a mouse model for p210^{BCR-ABL}+ leukemias, it was necessary to employ different promoters. Many groups have made use of alternative promoters such as metallothionein, *TEC* (hematopoietic specific) (100), immunoglobulin heavy chain enhancer (E μ), a tetracycline-repressible promoter (101), or the part of the long-terminal repeat (LTR) of the myeloproliferative sarcoma virus (MPSV), in order to generate transgenic mice (Table 1). Although these models developed hematological malignancies, all of them were exclusively diagnosed as ALL rather than granulocyte hyperplasia; much less frequently, a myeloid phenotype after a latency of 8 to 44 wk (100) was diagnosed.

Additional mouse models of *BCR-ABL* leukemogenesis have been generated based on homologous recombination. This approach has been used to fuse p190^{BCR-ABL} coding sequences into the endogenous *BCR* gene (102). After 4 mo, 95% of chimeric mice expressing one p190^{BCR-ABL} allele developed pre-B-ALL. This approach of producing transgenic *BCR-ABL* mice was able to replicate acute leukemia but no information is available on the use of this knock-in strategy to target p210^{BCR-ABL} at the *BCR* locus.

The most recent development in the hallmark of CML knock-in model mice consists of an inducible genetic model based on the specific expression, in hematopoietic stem cells, of *BCR-ABL* regulated by a mouse stem cell leukemia (SCL) enhancer (103).

Table 1
Genotype-Phenotype Correlations in Men and Mice When Human Cancer-Gene Defect Targets Primitive Stem Cells

Human genotype	Human phenotype	Mouse genotype (knock-in)	Mouse phenotype	Ref.
t(9;11)(p22;q23) (<i>MLL-AF9</i>)	AML	MI1 (exon 8)-AF9	Chimeric mice develop AML	182
t(9;22)(q34;q11) (<i>BCR-ABLp190</i>)	B-cell ALL	Bcr (exon 1)-ABL	Chimeric mice develop B-cell ALL	102
t(8;21)(q22;q22) (<i>AML1-ETO</i>)	AML	AML1 (exon 5)-ETO AML1 (exon 4)-ETO	Embryonic lethal (Het) Embryonic lethal (Het)	183,184
inv(16)(q13;q22) (<i>CBFB-MYH11</i>)	AML(M4E ₀)	Cbfb (exon 5)-MYH11	Embryonic lethal (Het)	181
t(7;22)(p22;q12) (Ews-ERG)	Ewing's sarcoma	Ews (exon 7)-ERG*	T-cell leukemias	190

*This rearrangement is induced by *Cre*-recombinase expression under the control of a lymphoid specific cassette.

These animals are able to develop a chronic-phase CML-like disease, making it easier to study the leukemogenic mechanism during disease initiation and progression.

3.5. Knock-Out Animal Models With CML Phenotypes

Consistent with the importance of RAS signaling in promoting growth of myeloid cells, JUNB, an antagonist of the RAS downstream target JUN and negative regulator of the cell proliferation and survival, was shown to act as a tumor suppressor in myeloid cells (104). *Junb*-null mice have severe vascular defects in the placenta, leading to early embryonic lethality. However, inactivation of JUNB, specifically in the long-term self-renewing hematopoietic stem cells (105), led to the development of a CML-like disease in mice, supporting the idea that CML originates from hematopoietic stem cells and that inactivation of JUNB could be important for CML development.

Gene-knockout studies, as it has been shown, have revealed key negative regulators of myelopoiesis, (e.g., JUNB). Moreover, mice with disruption of the gene encoding interferon consensus sequence binding protein (ICSBP or interferon regulatory factor 8) develop a chronic phase-CML like disease (106). Expression of this ICSBP protein is significantly decreased in mice with *BCR-ABL*-induced CML disease (107). These data indicate that ICSBP is a tumor suppressor and its downregulation is important for the pathogenesis of CML.

Many questions remain unanswered. One of these that is yet unresolved is the requirement of *BCR-ABL* expression for the development and maintenance of a *BCR-ABL*-induced leukemia. It is widely accepted that translocation of *BCR-ABL* is the primary oncogenic event shown to be essential for the development of Ph⁺ leukemias, but its maintenance function in the disease remains unsolved. In vitro experiments in *BCR-ABL* tetracycline-regulatable ES cell differentiation system have revealed that continual expression of *BCR-ABL* is required for the expansion of progenitors and myeloid cells (108); nevertheless, in vivo mouse models are not fully determinant in this final conclusion. Although a transgenic *BCR-ABL* tetracycline-regulatable B-ALL mouse model in which administration of tetracycline induces complete remission of the phenotype does exist (109), not all of the studied animal lines have confirm this pattern; some succumbed to a rapidly progressing B-cell leukemia that was independent of *BCR-ABL* expression after 2 to 4 wk of remission.

Altogether, it is clear that the available mouse models of *BCR-ABL* induced diseases do not closely mimic CML in humans and that a better understanding of the mechanism of disease progression will require generation of other transgenic lines in which *BCR-ABL* expression is under the control of promoters active only in primitive hematopoietic progenitors. The establishment of disease models of *BCR-ABL*-induced leukemia with a long myeloproliferative phase would be necessary for assessing the mechanisms of disease progression.

4. The Cellular Origin of the Human Disease

A complete understanding of the cancer process requires more detailed knowledge of the origins of neoplastic growth. Identifying the initial target cell origin of cancer and the respective contributions of different genetic events to cell-fate determination and disruption of local homeostasis would be crucial for the development of novel nontoxic therapies that influence tumor-cell behavior.

Two main hypotheses have been put forward to explain the origin of cancer. One view is that all tumors come from multipotent stem cells, as these have the long lifespan required for the accumulation of many genetic alterations that are found in most tumors. In this scenario, tumor-cell fate and behavior are determined by specific combinations of genetic or gene-expression changes that have occurred during tumor development (110). An alternative view is that there are a variety of target cells that range from stem cells to committed cells that have begun the process of cell determination and differentiation, the nature of the target cell being the important influence on malignant potential (111) (see Fig. 3). The parallelism described in past years between the mechanistic similarities of normal stem cells and cancer cells has led to the notion of “cancer stem cells” (110,112,113) as the original cells in which cancer begins.

Leukemia provides the best evidence that normal stem cells are the targets of transforming mutations (66,114). The cells capable of initiating human acute myeloid leukemia (AML) in NOD/SCID mice have a $CD34^+CD38^-$ phenotype (a population enriched for hematopoietic stem cells [HSCs]), phenotype similar to normal HSCs (115,116). Conversely, $CD34^+CD38^+$ leukemia cells can not transfer disease to mice in the vast majority of cases (see Fig. 4). In CML, leukemia-associated chromosomal rearrangements have been also found in $CD34^+CD38^-$ cells (117–119). All these suggest that HSCs, rather than committed progenitors, are the target for leukemic transformation.

Aside from the studies that point to a stem cell origin, other authors have suggested that the Ph^+ rearrangement might happen in stem cells more primitive than $CD34^+CD38^-$ cells (i.e., it may occur at the level of putative hemangioblasts [120]). The identification of the *BCR-ABL* fusion gene in various proportions of endothelial cells generated from CML patients' bone marrow cells in vitro has led to theorization that CML might originate from bone marrow derived hemangioblastic precursor cells. Indeed, recent studies have provided direct evidence that cells capable of initiating human CML have a differentiation potential similar to hemangioblasts (121), which suggests that hemangioblasts, rather than HSCs, are the target for leukemic transformation in CML.

These advances, which have recently extended to the study of breast cancer (122) and glioblastoma (123), indicate that many types of cancer cells can be organized into hierarchies, ranging from malignant cancer stem cells, which have extensive proliferative potential, to differentiated cancer cells, which have limited proliferative potential. In conclusion, all results indicate that cancer can be considered a disease of unregulated self-renewal in which genome alterations convert normal stem cell self-renewal pathway into engines for neoplastic proliferation (124).

5. CML Treatment Points to *BCR-ABL* Targeted Therapy

CML is a clonal proliferative malignancy originating from a pluripotent HSC. Until recently, the therapeutic armamentarium for CML was limited to allogeneic stem cell transplantation (SCT), conventional chemotherapy with agents such as busulfan (BUS) and hydroxyurea (HU), and treatment with interferon (IFN)- α based regimens (125). All of these options have major drawbacks with respect to efficacy and tolerability. Currently, allogeneic SCT is the only treatment with known curative potential in CML. However, most patients are not eligible for this therapy because of advanced age or lack of a suitable stem-cell donor (126), there is a significant risk of treatment-related morbidity

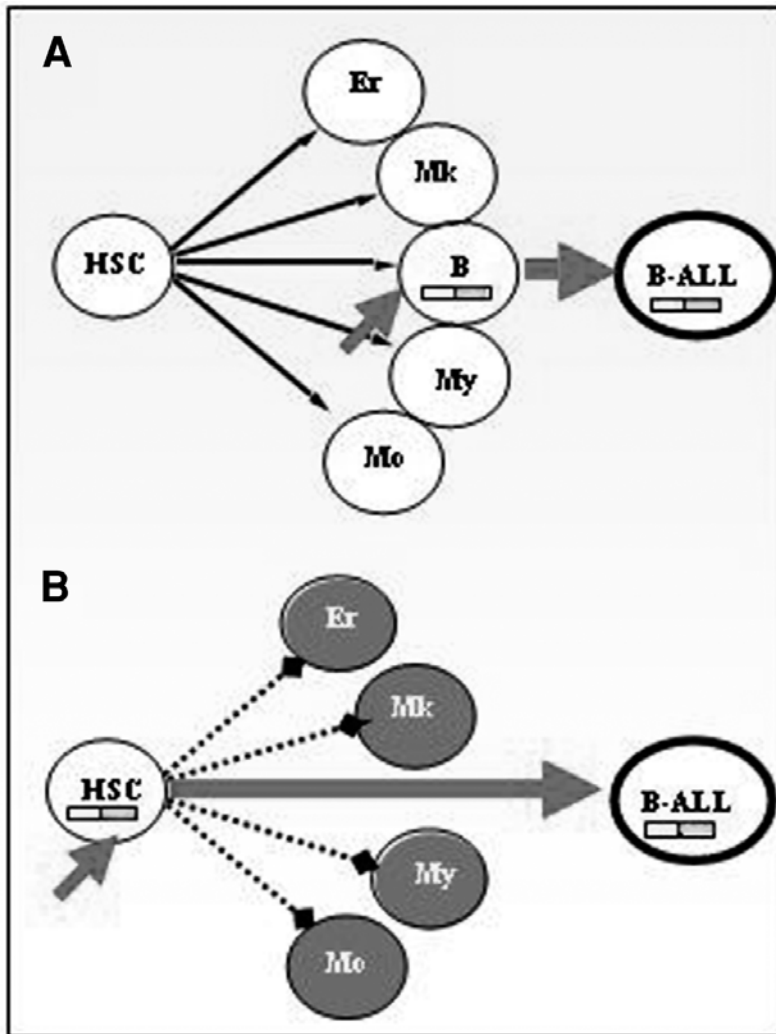


Fig. 3. Proposed model for the role of *BCR-ABL*^{p190}, in particular, and chromosomal abnormalities, in general, in cell lineage specification. **(A)** Adopting a lineage among two or more options is a fundamental developmental decision in multicellular organisms. In the context of the hematopoietic system, a small pool of multipotent stem cells maintains the multiple cell lineages that constitute blood. Ph-ALL has been thought to affect progenitor cells committed to B-cell lymphoid differentiation. **(B)** The demonstration that normal uncommitted progenitor cells are the target for leukemic transformation in Ph-ALL, indicate that genes created by chromosomal abnormalities into the target stem cell are candidate instigators of lineage choice decisions. Consistent with this, forced expression of these genes can, in certain cellular environments, select or impose a lineage outcome (66,114).

and mortality. IFN- α treatment leads to both hematological and cytogenetic response in many chronic phase CML patients (127) and its effectiveness at treating a significant portion of CML patients suggests that IFN- α therapy may have some selectivity toward destroying Ph⁺ leukemic cells or inhibiting the oncogenic activity of BCR-ABL. On the

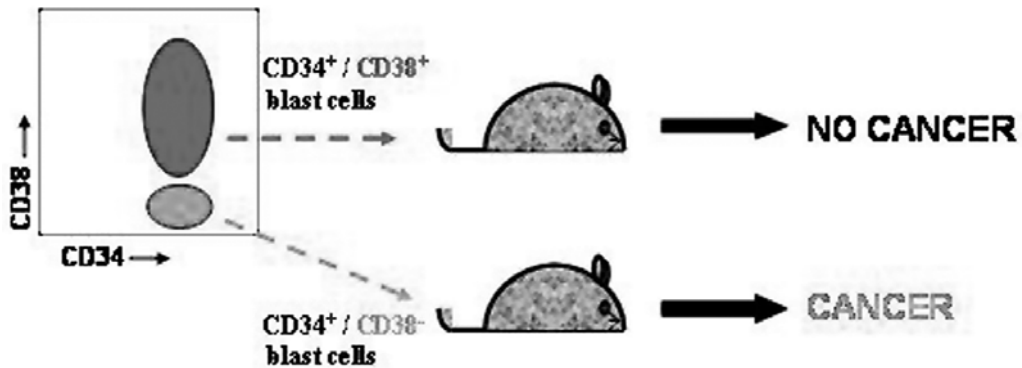


Fig. 4. Nature of the leukemic target cell. Leukemia provides the best evidence that normal stem cells are the targets of transforming mutations. Several assays in AML (*115,116*) and CML (*117–119*) have shown that cells capable of initiating leukemia in NOD/SCID mice have a CD34⁺CD38⁻ phenotype, similar to normal HSC. Nevertheless, CD34⁺CD38⁺ cells are not able to transfer disease to mice, suggesting that HSCs are the target for leukemic transformation.

other hand, up to 20% of CML patients do not tolerate IFN- α therapy because of toxicity problems (*128*).

Until recently, the use of nonselective cytotoxic drugs that affect normal and malignant cells has been the habitual treatment for many cancers, including Ph⁺ leukemias. Because most available chemotherapeutic agents show some degree of S-phase specificity, cells that are not actively dividing, as stem and progenitor cells, have shown resistant to such drugs. This raises the possibility that the quiescent leukemic cells identified in CML patients are likely to survive standard chemotherapy regimens. For this and other reasons, novel approaches in the Ph⁺ leukemias treatment are being targeted to the BCR-ABL tyrosine kinase. BCR-ABL fusion oncoprotein is present in almost all patients with CML and is expressed at high levels only in the leukemic cells. In addition, because the mitogenic and antiapoptotic effects are dependent on the tyrosine kinase activity of BCR-ABL, inhibition of its activity triggers growth arrest and apoptosis of the leukemic cells.

Efforts to inhibit the function of BCR-ABL have been performed since the early 1990s. Because BCR-ABL is a tyrosine kinase protein, development of molecular targeted therapy has been directed toward inhibiting this tyrosine kinase activity. More than 100 oncogenes have been defined that contribute to carcinogenesis (*129,130*); tyrosine kinases represent a large fraction of these known dominant oncogenic proteins (*130*). However, the high degree of commonality among kinase ATP-binding regions (*131*) suggests that it would be difficult to develop compounds that specifically inhibited a single or limited set of tyrosine kinases without having cross-reactivity toward others.

The identification of naturally occurring compounds with specific inhibitory activity toward tyrosine kinases such as Herbimycin A (*132*), Genistein (*133*), or Erbstatin (*134*), and the capacity of these to inhibit BCR-ABL cellular transformation (*135,136*), suggest that inhibition of deregulated ABL tyrosine kinase activity may be a viable therapeutic approach toward ABL-induced leukemias. However, the effect of decreased tyrosine

kinase activity was due more to activation of the proteasome 20S than to a specific inhibition of the kinase domain itself. More selective inhibition was obtained by drugs named tyrphostins, from tyrosine phosphorylation inhibitors ([137,138](#)), which are more specific for individual tyrosine kinases but with a limited specificity and potency at the cellular level.

Novel treatments strategies for BCR-ABL+ leukemias can be divided, currently, in two groups—treatments centered in downregulation of intracellular BCR-ABL protein levels and those focused in the inhibition of BCR-ABL tyrosine kinase activity. The introduction, as part of this last group, of imatinib mesylate (Gleevec, Glivec formerly STI571; Novartis Pharma AG; Basel, Switzerland), a potent and specific inhibitor of the BCR-ABL tyrosine kinase ([139](#)), has caused a rapid change in the management of patients with CML and, although it is presently indicated as first-line therapy for CML in all phases, interest in identifying new BCR-ABL inhibitors is increasing. In 2000, the novel pyrido (2,3-*d*) pyrimidine derivative, PD180970, originally identified as a Src tyrosine kinase inhibitor ([140](#)), was also shown to potently inhibit BCR-ABL tyrosine kinase activity and induce apoptosis of Ph⁺ leukemic cells ([141](#)). Treatment with PD180970 inhibits the phosphorylation of Gab2 and Crkl, affecting neither the viability nor growth of BCR-ABL negative human leukemic cells ([141](#)). These results highlight PD180970 as a promising therapeutic agent against BCR-ABL Ph⁺ leukemia. Finally, and consistent with in vitro and pharmacokinetic profile, a novel selective inhibitor of BCR-ABL, AMN107, has been described that is significantly more potent than imatinib and active against a number of imatinib resistant BCR-ABL mutants ([142](#)). Crystallographic analysis of ABL-AMN107 complexes has provided a structural explanation for the differential activity of this molecule and imatinib against imatinib-resistance BCR-ABL, standing out as a promising new inhibitor for the therapy of CML and Ph⁺ ALL.

6. Clinical Trials With Gleevec

Searching molecules with tyrosine kinase inhibitory activity led to the identification of imatinib mesylate (STI571, Gleevec). Initially, Gleevec was developed as a specific inhibitor of the platelet derived growth factor receptor tyrosine kinase (PDGF-R) ([143,144](#)) and a suppressed tumor growth of PDGF-R activated cell lines in vivo. Surprisingly, this compound not only inhibited PDGF-R tyrosine kinase activity but quantitatively inhibited not only all ABL tyrosine kinases (including the 210-kD BCR-ABL and the 190-kD BCR-ABL [[144](#)]), but also the c-kit tyrosine kinase (the receptor for stem cell factor) ([143,145](#)), and ARG ([146](#)).

The selective inhibition of BCR-ABL by imatinib was shown to be mediated via interaction between the inhibitor and the aminoacids constituting the ATP binding cleft of the tyrosine kinase. Imatinib competitively binds to the ATP binding site ([147](#)), inhibiting the tyrosine phosphorylation of its substrates and the growth of the affected leukemic cells. Imatinib acts by downregulating antiapoptotic XIAP, cIAP1, and Bcl-xL, without affecting Bcl-2, Bax, Apaf-1, Fas (CD95), Fas ligand, and BCR-ABL levels ([148](#)). Imatinib also inhibits STAT5, Akt kinase, and NF-κB activities in the BCR-ABL positive cells ([149](#)).

The high selectivity of imatinib mesylate at suppressing BCR-ABL-induced cell expansion in vitro and in vivo along with imatinib mesylate's pharmacological properties ([150](#)) have led to the testing of this in CML patients refractory to INF-α therapy.

6.1. Gleevec Treatment in CML Patients

In 1998, a phase I study of imatinib mesylate treatment on chronic phase CML patients refractory or resistant to interferon-based therapy began. When these patients became non-responsive to IFN- α therapy, no treatment options proven to be effective against CML were available. Druker, Talpaz, Sawyers, and colleagues reported that 53 of 54 patients had complete hematologic responses after 4 wk of imatinib mesylate treatment (151), most of them maintaining normal blood counts and complete hematological responses for over 1 yr (151).

The success of imatinib mesylate treatment on chronic phase CML patients in phase I studies led to large-scale phase II and III studies, reinforcing the positive results from phase I. Even more impressive, in a randomized phase III study of 553 patients not previously treated with IFN- α , only 1.5% of imatinib mesylate-treated patients had disease progression (152).

6.2. Gleevec Treatment in Acute Leukemia and Blast Crisis

The high rate of remission in imatinib mesylate-treated chronic phase CML patients treated led to expansion of the study to determine the effect of imatinib mesylate therapy on blast crisis CML patients and Ph⁺ B-ALL patients. However, only 18% of patients had responses and the remaining 20 imatinib mesylate treated patients with lymphoid blast crisis CML or Ph⁺ B-ALL had a similar lower level of disease suppression compared with imatinib mesylate treated chronic phase CML patients (153). During the aggressive phase of CML blast crisis, numerous secondary genetic aberrations, including duplication of the Ph chromosome, were common (154). These findings suggested that failure of imatinib mesylate treatment in blast phase CML patients could result from either the presence of added oncogenic events beyond the Ph chromosome or a heightened level of BCR-ABL tyrosine kinase activity ineffectively suppressed by the dose of imatinib mesylate. If duplication of the Ph chromosome was the reason for this low response, then higher doses of imatinib mesylate could be effective to suppress the ABL tyrosine kinase activity. Treatment showed that imatinib mesylate at a higher dose of 800 mg/d is toxic, limiting the therapeutic applicability of imatinib mesylate in treating CML patients expressing higher amounts of BCR-ABL.

6.3. Mechanisms of Resistance to Gleevec

The presence of BCR-ABL mutants resistant to or with reduced sensitivity to Imatinib mesylate inhibition appears to be the most frequent mechanism by which CML patients relapse from Imatinib mesylate therapy (155–157). More than 80% of blast phase CML cases have definable additional genetic aberrations including trisomy 8, i(17q) (31), loss of p53 function (33), MYC amplification (34), RB deletion/rearrangement (35), and p16INK4A rearrangement/ deletion (36). These findings suggested that the high rate of relapse in blast crisis CML patients treated with imatinib mesylate could result from the acquisition of additional oncogenic events that render Ph⁺ leukemic cell growth independent of BCR-ABL tyrosine kinase activity. Surprisingly, the large majority of accelerated and blast crisis Ph⁺ leukemic cells from imatinib mesylate-resistant CML patients maintained high levels of BCR-ABL tyrosine kinase activity.

To determine how imatinib mesylate achieves its high specificity at inhibiting BCR-ABL transformation, the crystal structure of the kinase domain of ABL complexed

with imatinib mesylate was resolved. A critical component in determining the inactive vs active conformation of tyrosine kinases is the orientation and phosphorylation status of residues in the activation loop of the kinase. The initial search for BCR-ABL mutants resistant to imatinib mesylate focused on the identification of mutants in the ABL kinase domain predicted to obstruct direct imatinib mesylate binding. The ABL kinase T334I mutant was described as the most frequent BCR-ABL mutant in CML blast crisis patients resistant to imatinib mesylate therapy (155). Numerous additional BCR-ABL mutants resistant to imatinib mesylate have subsequently been identified in CML patients (156,157). Using a random mutagenesis approach, a range of BCR-ABL mutants outside of the ABL kinase domain, in the cap, SH3, and SH2 domains of ABL were identified as resistant to imatinib mesylate inhibition (158). These domains play pivotal roles in the transition from an inactive to active conformation of ABL. Because imatinib mesylate binds to the inactive conformation of ABL, mutations in these ABL domains could be predicted to abrogate imatinib mesylate binding.

The BCR portion of BCR-ABL has not been sequenced in BCR-ABL clones resistant to imatinib mesylate treatment and different regions of BCR are critical for BCR-ABL activation. So mutations in BCR that up-regulate BCR-ABL tyrosine kinase activity may also be relatively resistant to imatinib mesylate. Some imatinib mesylate resistant patient samples with an activated BCR-ABL tyrosine kinase domain contain no mutations in the ABL kinase domain (159). Sequencing BCR-ABL in its entirety in these samples may identify novel mutations in unexpected regions of BCR-ABL that render the protein insensitive to imatinib mesylate treatment.

BCR-ABL expression has been shown to down-regulate proteins involved in DNA repair. Mutation in the xeroderma pigmentosum group B protein results in a human DNA repair disorder, and BCR-ABL expression inactivates this enzyme by binding to and phosphorylating the enzyme (160). BRCA1 deficiency in mice leads to heightened sensitivity to DNA double-strand breaks (161), and this protein is down-regulated upon BCR-ABL induction in multiple cell types (162–164). Determining which DNA repair mechanism is largely responsible for the mutator phenotype present in BCR-ABL expressing cells may identify therapeutic approaches that could suppress the generation of imatinib mesylate-resistant BCR-ABL mutants in CML patients.

6.4. *Ph⁺ Cell Populations that are not Eliminated by Gleevec*

The origin of CML begins in a HSC, a target population that is largely quiescent (165). In vitro and in vivo studies in the last years have shown that these quiescent Ph^+ HSCs, origin of CML, are insensitive to imatinib mesylate treatment (166,167), not been this cells eliminated in CML patients.

Imatinib mesylate inhibits malignant primitive progenitor growth primarily through inhibition of their abnormally increased proliferation rather than selective induction of apoptosis (168). This suggests that BCR/ABL kinase activity may be required for abnormal proliferation and expansion of Ph^+ cells in CML but may not be essential for preservation of primitive malignant cells. BCR-ABL kinase inhibition by imatinib mesylate may therefore remove the proliferative advantage of Ph^+ progenitors and their progeny cells allowing regrowth of coexisting Ph^- cells without eliminate all Ph^+ primitive progenitors.

In conclusion, although imatinib mesylate therapy is able to suppress the oncogenic activity of wild-type *BCR-ABL* expression, the treatment is unable to destroy all Ph⁺ cell populations. As in Ph⁺ HSCs, the inability of imatinib mesylate to eliminate Ph⁺ progenitors suggests that CML patients will require long-term imatinib mesylate monotherapy, which may lead to the generation and selection of *BCR-ABL* escape mutants resistant to the inhibitor.

7. New Therapeutic Options

Although imatinib mesylate produces high rates of complete clinical and cytogenetic responses in the chronic phase, resistance is universal and clinical relapse develops rapidly in the advanced phases of the disease. Because of alternative mechanisms driving the growth and survival of the malignant clones could be responsible for imatinib resistance, novel tyrosine kinase inhibitors that target BCR-ABL as well as agents that down-regulate BCR-ABL levels, regardless of wild-type or mutant status, may need to be developed for the future therapy of CML.

In recent years, different therapeutic options have been developed in order to offer other possibilities to imatinib resistance BCR-ABL leukemias.

7.1. Alternative Small Molecules Inhibitors

Small molecules inhibitors belonging to the class of pyrido(2,3-d)pyrimidine compounds (PD 166326, PD 173995 and PD 180970) have been shown to selectively suppress the tyrosine kinase activity of small group of proteins including BCR-ABL (169–171). These compounds required about 10-fold less drug compared with imatinib to inhibit BCR-ABL tyrosine kinase at similar levels suggesting to be useful at treating Ph⁺ patients resistant to the highest doses of imatinib because of high levels of BCR-ABL expression.

7.2. Inhibiting BCR-ABL mRNA Production

Inhibitor of *BCR-ABL* production at the mRNA level via antisense oligonucleotide, small interfering RNA, peptide nucleic acid, or ribozyme expression of BCR-ABL junction sequences effectively suppresses BCR-ABL in vitro transformation (77,172–178). However, these strategies are limited by the lack of proven technologies to introduce sufficient amount of such agents into a patient and thereby suppress the leukemia.

7.3. Suppressing BCR-ABL Protein Levels

Heat shock proteins (HSP) help proteins avoid misfolded, inactive, or aggregated states (179). Inactivation of Hsp90 using benzoquinone ansamycins such as geldanamycin or 17-AAG results in a rapid degradation of the client proteins of Hsp90, BCR-ABL for example. Consistently with this, geldanamycin selectively increases apoptosis of BCR-ABL expressing cells (180,181) without severely affecting normal protein translation.

Arsenic trioxide, in the same way, is able to decreased BCR-ABL protein levels but, in this case, inhibiting *BCR-ABL* translation (182).

7.4. Obstruction of Signaling Components Critical for BCR-ABL Oncogenesis

Imatinib has been shown to elicit MAPK activation in imatinib-resistant, Ph⁺ leukemic cells. Interruption of this pathway in conjunction with imatinib is associated

with a highly synergistic induction of mitochondrial damage and apoptosis (183). Farnesyl transferase inhibitors (SCH66336), which are known to block Ras mediated signaling, have been shown to inhibit cell growth of BCR-ABL positive leukemic cells and, in combination with imatinib, inhibit synergistically colony growth of primary CML, causing apoptosis of imatinib resistant Ph⁺-expressing cells.

In summary, accumulating experience with treatment of advanced stages of CML with imatinib has indicated that resistance to this agent is common. Novel BCR-ABL targeted agents that inhibit its tyrosine kinase activity or reduce its intracellular levels hold great promise in preventing and treating imatinib-resistance disease.

8. Conclusions

The origin of the translocation *BCR-ABL* begins in a stem cell. The BCR-ABL story has demonstrated that identifying and selectively targeting the primary oncogenic event essential for the development of Ph⁺ leukemias is sufficient to suppress the cancer. BCR-ABL expression has been shown to regulate a plethora of signaling molecules, and it is unclear whether inhibition of any single signal transduction pathway would be sufficient to block leukemogenesis. The discovering of imatinib as a potent inhibitor of the tyrosine kinase activity BCR-ABL and its powerlessness in the inhibition of the Ph⁺ HSC, has led, out of necessity, to the identification of other important factors in the pathogenesis of CML and to design alternative treatments strategies. Therapeutic approaches aimed at suppressing the leukemic activity of a Ph⁺ cell by imatinib mesylate-like mechanisms are unlikely to destroy all cancerous cells in a patient and would most likely require life-long treatment. Eliminating the cellular origin of leukemia is the only curative approach for the disease; in CML, this would require destroying the Ph⁺ HSC that is largely quiescent. The well-understood pathogenesis of Ph⁺ leukemias is turning out to be fertile ground for the testing of novel therapies. Defining the effectiveness of these treatment approaches against imatinib mesylate resistant patients may help guide the next wave of cancer therapeutics.

Acknowledgments

We thank all the members of lab 13 for their helpful comments and constructive discussions on the manuscript. Research in our group was supported by MEyC (BIO2000-0453-P4-02, SAF2003-01103, and FIT-010000-2004-157), Junta de Castilla y León (CSI06/03), ADE de Castilla y León (04/04/SA/0001), FIS (PI020138, G03/179, and G03/136), and USAL-CIBASA project. MPC is a recipient of a MCyT fellowship.

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Angiogenesis and Cancer

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Summary

Angiogenesis, the creation of neovasculatures from pre-existing ones, is required for various physiological processes. However, pathological angiogenesis is a hallmark of malignant tumors, metastasis and various ischemic as well as inflammatory disorders. Angiogenesis is regulated by the balance between proangiogenic factors and antiangiogenic factors, and concentrated effort in this area of research has led to the discovery of a growing number of angiogenesis-associated factors and the complex interactions among these factors. Understanding of the regulatory mechanisms of these factors in mediating the angiogenic process involved in tumor growth prompted the application of antiangiogenic factors on experimental tumor models with successful outcomes. Based on these experimental results, some antiangiogenic agents have been tested in clinical trials. In this review, the process and regulators of angiogenesis, the involvement of angiogenesis in cancer development and the application of antiangiogenic therapies on established tumors would be discussed. Among various antiangiogenic reagents, special emphasis will be given to antiangiogenic reagents derived from vascular basement membranes, a crucial regulator of angiogenesis, rather than a structural tissue component.

Key Words: Angiogenesis; endothelial cell; blood vessels; tumor; vascular basement membrane; endostatin; tumstatin; integrin; VEGF; angiopoietins.

1. Introduction

Angiogenesis, the formation of new capillaries from preexisting blood vessels, is required for several physiological processes as well as pathological conditions. In recent years, the involvement of angiogenesis in the development of malignant tumors and metastasis has been extensively studied. Therapeutic strategies to suppress angiogenesis has been examined on experimental tumor models with successful outcomes. Based on these experimental data, some antiangiogenic agents have been tested in clinical trials. In this review, the process and regulators of angiogenesis, the involvement of angiogenesis in cancer development, and the application of antiangiogenic therapies on established tumors will be discussed. Among various antiangiogenic reagents, special emphasis will be given to antiangiogenic reagents derived from vascular basement membranes.

2. The Process of Angiogenesis

Angiogenesis, the formation of new blood vessels from preexisting ones, is composed of several steps (*see Fig. 1*): (1) the degradation of vascular basement membrane matrix by protease, (2) migration and proliferation of endothelial cells into interstitium, (3) endothelial tube formation, (4) recruitment and attachment of mesenchymal cells to

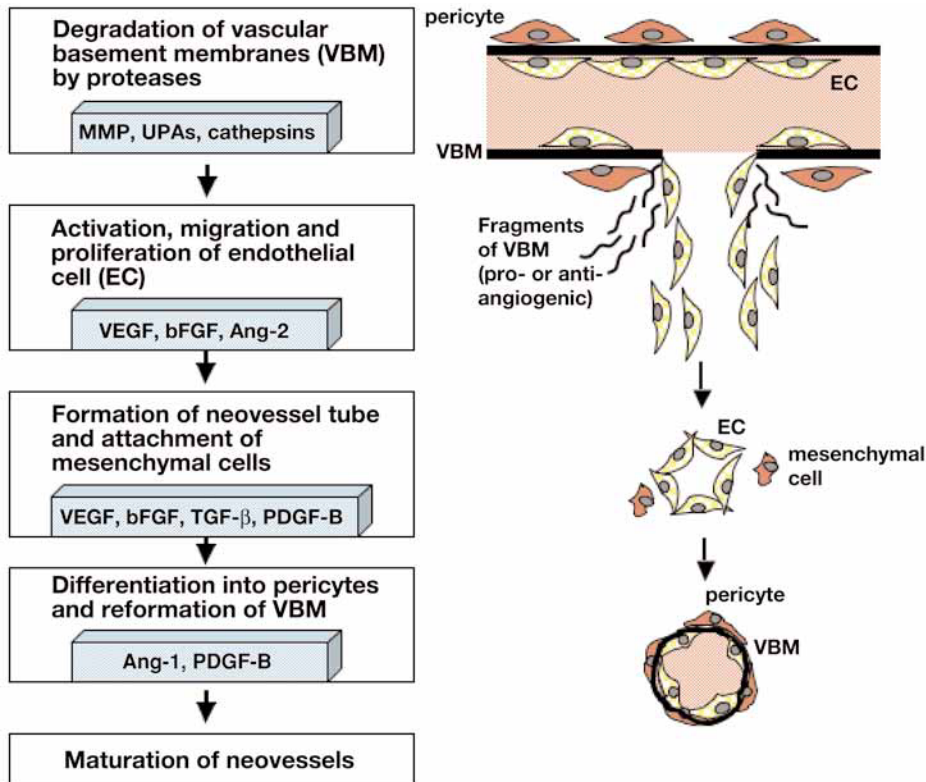


Fig. 1. The vascular microenvironment in the process of angiogenesis. Vascular basement membranes (VBM) are degraded by proteases such as MMPs in activated blood vessels. Proangiogenic growth factors (e.g., VEGF, bFGF, PDGF) are released from VBM, produced by surrounding tumor cells, fibroblasts, and immune cells, and induce proliferation and migration of endothelial cells (EC). Fragments of VBM have pro- or antiangiogenic activities and regulate angiogenesis. Formation of EC tube with the attachment of pericytes leads to the maturation of neovessels with VBM.

the endothelial cell tube, and (5) maturation of blood vessels with the formation of vascular basement membrane (1). In these angiogenic steps, several angiogenic factors and extracellular matrix (ECM) proteins are involved. Although small blood vessels consist only of endothelial cells, larger vessels are surrounded by mural cells such as pericytes and smooth muscle cells. In the quiescent stage, endothelial cells reside on vascular basement membranes mainly composed of type IV collagen, laminin, nidogen, and heparan sulfate proteoglycan (HSPG) (2). Upon angiogenic stimuli, proteases represented by matrix metalloprotease (MMP) are activated and, in turn, degrade vascular basement membranes, thus leading to the migration of activated endothelial cells into interstitium. Proteinases also expose new cryptic epitopes and produce fragments of ECM proteins such as type IV collagen, potentially leading to the regulation of migratory capacity of endothelial cells as well as smooth muscle cells (3). A provisional matrix of fibronectin, fibrin, and other components provides a supporting scaffold, guiding endothelial cells to their targets and thus facilitating neovessel formation. Proangiogenic growth factors, as well as these provisional matrix proteins, affect the

migration, proliferation, and tube formation of endothelial cells via specific cell surface receptors for those growth factors as well as integrin families. Integrin receptors are composed of heterodimers of α and β subunit and display a variety of biological functions upon binding to various matrix proteins and peptides, assisting vascular cells to build new vessels in coordination with their surrounding matrix environments (4,5). Among various integrin families, the $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins have been extensively investigated for possible involvement in angiogenic process, and pharmacological blockade of these integrins has been reported to suppress pathological angiogenesis (6,7). However, genetic deletion studies of the $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins suggest that integrins expressed on vascular endothelial cells inhibit angiogenesis by suppressing vascular endothelial growth factor (VEGF)-mediated endothelial cell survival, through transdominant effect on other proangiogenic integrins or by mediating the antiangiogenic activity of thrombospondin-1 and other angiogenesis inhibitors (4,8,9). The possible positive or negative roles of integrins on angiogenesis depending on specific conditions thus require further clarification.

Recent advances in molecular biology have further revealed the involvement of antiangiogenic factors in regulating angiogenesis (10,11). The balance between proangiogenic factors and antiangiogenic factors is considered to be critical in regulating angiogenic process. Thus, excess amount of proangiogenic stimuli leads to the progression of angiogenesis, but excess antiangiogenic milieu results in the resolution of angiogenic process. Various factors are known to induce angiogenesis (Table 1), but among those, proangiogenic capacity of VEGF, basic fibroblast growth factor (bFGF) and angiopoietins are well investigated and established to date. At present, there are many known antiangiogenic factors (Table 2) but these factors were already investigated extensively following the discovery of endostatin, a C-terminal domain of type XVIII collagen, exhibiting a potent antiangiogenic antitumor effect (12). Subsequently various intrinsic antiangiogenic factors have been reported including angiostatin, restin, arresten, canstatin, and tumstatin (13–18).

Endothelial cells differentiate from angioblasts in the embryo and from endothelial progenitor cells (EPC), multipotent adult progenitor cells or side-population cells in the adult bone marrow (19,20). A portion of angiogenic factors are also known to recruit endothelial progenitor cells and facilitate the formation of neovessels (21). Endothelial and hematopoietic progenitors share common markers and are affected by common signaling pathways. In fact, hematopoietic stem cells (HSCs) and leukocytes stimulate angiogenesis via releasing angiogenic factors or by transdifferentiation to endothelial cells (22,23). The involvement of VEGF, placental growth factor (PlGF), angiopoietin-1, and inhibitor of differentiation (Id) in this process had been reported (24,25). Angiogenesis is involved in a variety of physiological and pathological conditions, but initially it plays a critical role in the development of blood vessels and the cardiovascular system. Vasculogenesis, neovessel formation originating from EPCs, is critically involved in the development and is tightly linked to angiogenesis. Recent evidence indicates that EPCs contribute to angiogenic process also in the adult with ischemic, malignant, or inflammatory disorders. Therapeutic efficacy of EPC to stimulate vessel growth in ischemic disorders has been reported (26,27). Angiogenesis and arteriogenesis refer to the sprouting and subsequent stabilization of these sprouts by mural cells. Angiogenesis is involved in physiological settings such as ovarian cycle, endometrium

Table 1
Proangiogenic Factors

VEGF	PDGF-BB
bFGF	Midkine
PlGF	Pleiotrophin
G-CSF	TGF- α , β
HGF	Follistatin
Angiopoietin-1/2	IL-3, 8
PD-ECGF	TNF- α
Angiogenin	Proliferin

Table 2
Antiangiogenic Factors

IFN- α , β , γ	Kringle 5
PF4	PEDF
Prolactin 16 kD frag.	IL-4, 10, 12
Angiostatin	Troponin-1
Endostatin	IP-10
Vasostatin	MIG
Arresten	Thrombospondin-1
Canstatin	Kininostatin
Tumstatin	TIMPs
Vasohibin	PEX
Angioarrestin	PAI
Endorepellin	Meth-1, 2

remodeling, and wound healing. Excessive angiogenesis is known to cause various disorders, and the best known are cancer, rheumatoid arthritis, psoriasis, and visual disturbance represented by diabetic retinopathy and retinopathy of prematurity (11). Recent findings further added common disorders such as atherosclerosis, obesity, bronchial asthma, infectious disease, and endometriosis to the growing list of diseases associated with the involvement of angiogenesis. Several congenital and inherited diseases, such as DiGeorge syndrome, are also caused by abnormal vascular remodeling and angiogenesis. Insufficient angiogenesis and abnormal vessel regression lead to hypertension, preeclampsia, neurodegeneration as well as heart and brain ischemia.

3. Factors Involved in Regulating Angiogenesis

3.1. VEGF

The role of VEGF (also termed as VEGF-A) in regulating angiogenesis has been intensely investigated, and VEGF-signaling is critically involved in physiological as well as pathological angiogenesis represented by tumor growth (28). A gene family of VEGF consists of VEGF-A, VEGF-B, VEGF-C, and PlGF (28). VEGF-A is a key regulator of blood vessel growth, and VEGF-C and VEGF-D are involved in regulating lymphatic angiogenesis (29). Inactivation of a single *Vegf* allele in mice resulted

in embryonic lethality between days 11 and 12 as a result of numerous developmental anomalies, defective vascularization in several organs, and reduction of nucleated red blood cells within the blood islands in the yolk sac, suggesting an essential role of VEGF in embryonic vasculogenesis and angiogenesis (30,31). Mice deficient in VEGF-B or PlGF (32) did not exhibit any evident developmental abnormalities. VEGF induces the proliferation of endothelial cells in vitro (28), and induces angiogenic response in vivo (33). VEGF also induces survival of endothelial cells partly mediated via activating phosphatidylinositol (PI)-3 kinase-Akt pathway and by the increased production of antiapoptotic proteins Bcl-2 and A1 (34,35). VEGF also promotes monocyte chemotaxis and induces vascular permeability, leading to inflammation and other pathological circumstances (36,37). Consistent with its role in regulating vascular permeability, VEGF also induces fenestration of endothelial cells in some vascular beds (38).

Four different splice variant isoforms of VEGF (VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉, VEGF₂₀₆) have been reported (39,40). The properties of native VEGF (45 kDa) closely correspond with those of predominant isoform, VEGF₁₆₅, which is secreted but a significant fraction remains bound to the cell surface and ECM (41). Expression of VEGF mRNA is induced under hypoxic condition in endothelial cells, mediated via hypoxia-inducible factor (HIF)-1 (42). In addition, growth factors such as transforming growth factor (TGF)- α , TGF- β , insulin-like growth factor (IGF)-1, basic fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) upregulates the expression of VEGF mRNA (28). Inflammatory cytokines such as interleukin (IL)-1 α and IL-6 induce the expression of VEGF mRNA in synovial fibroblasts, leading to the development of joint lesions in rheumatoid arthritis.

VEGF binds to tyrosine kinase receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) (28), and VEGF-C and VEGF-D bind to VEGFR-3 (29). VEGF also bind to a family of co-receptors, the neuropilins.

3.1.1. VEGFR-1 (Flt-1)

Not only VEGF-A, but also VEGF-B and PlGF, which do not bind to Flk-1, bind to Flt-1. Upon binding to VEGF, Flt-1 undergoes weak tyrosine autophosphorylation (43). A soluble extracellular domain of Flt-1 (sFlt-1) serves as a VEGF inhibitor (44). Recent studies have revealed a synergistic effect of VEGF and PlGF in vivo as evidenced by impaired tumorigenesis in PlGF deficient mice (32). Flt-1^{-/-} mice die *in utero* between days 8.5 and 9.5 resulting primarily from excessive proliferation of angioblasts, suggesting that Flt-1 negatively regulates the activity of VEGF at least during early development (45). The role of Flt-1 in hematopoiesis and recruitment of endothelial progenitors have also been demonstrated (23). PlGF promoted collateral vessel growth in a model of myocardial infarction and treatment with anti-Flt-1 inhibited pathological conditions such as tumor, rheumatoid arthritis, and atherosclerosis suggest the involvement of Flt-1 in mediating angiogenic response (25).

3.1.2. VEGFR-2 (KDR/Flk-1)

VEGFR-2, a high-affinity receptor of VEGF, serves as a major mediator of VEGF in inducing mitogenesis, angiogenesis, and vascular permeability (46). Flk-1-null mice die *in utero* between days 8.5 and 9.5 resulting primarily from a lack of vasculogenesis and

failure to develop blood islands and organized blood vessels, suggesting the pivotal role of Flk-1 in developmental angiogenesis and hematopoiesis (47). Upon binding of the ligand, Flk-1 undergoes dimerization, tyrosine phosphorylation, and phosphorylates several proteins in endothelial cells such as phospholipase C- γ , PI-3 kinase, Ras GTPase-activating protein, and the Src family of tyrosine kinases (48,49). VEGF induces endothelial cell growth by activating the Raf-Mek-Erk pathway via protein kinase C, but not via Ras (50). Activation of Flk-1 is required for the effect of VEGF in preventing apoptosis of endothelial cells through activating PI-3 kinase-Akt signaling pathway (34).

3.1.3. Neuropilin

Neuropilin binds the semaphorin family and is implicated in neuronal guidance, and enhances VEGF₁₆₅-mediated chemotaxis (51). It binds to VEGF₁₆₅ in an isoform-specific manner, presents VEGF₁₆₅ to VEGFR-2 enhancing the signaling mediated via VEGFR-2 (51). Neuropilin-1-null mice exhibit embryonic lethality demonstrating the role of neuropilin-1 in the development of vascular system (52).

3.2. Angiopoietins

The angiopoietin family comprises three structurally related proteins, Angiopoietin (Ang)-1, Ang-2, and Ang-3/4 (53). Angiopoietin-1 consistently activates and phosphorylates Tie-2 receptor, but Ang-2 inhibits activation of Tie-2 and can even specifically block Ang-1-dependent phosphorylation (54). Mice deficient in Ang-1 die at embryonic day 12.5, exhibiting some defects in vascular maturation, suggesting the important role of Ang-1/Tie-2 signaling in inducing endothelial-matrix interactions (55). Mice deficient in Ang-2 die 2 wk after birth, exhibiting some defects in retinal vascularization and lymphatic function (56). The expression of Ang-2 has been reported to be up-regulated by hypoxia, hypoxia inducible factor (HIF)-1 α , VEGF, angiotensin-II, leptin, and other factors (57,58). In contrast, very little is known about the regulatory mechanism of Ang-1. Ang-1 tightens vessels by affecting junctional molecules (59), and is involved in the attachment of mesenchymal cells to endothelial-tube and differentiation to “pericytes,” resulting in mature, “nonleaky” blood vessels (55). Ang-1 binds to Tie-2 and promotes the firm attachment of pericytes (55). The biological roles of angiopoietins are, however, pleiotropic and context-dependent. Ang-1 induces vessel growth in skin, ischemic limbs. and in some tumors (60), possibly because of its effect on endothelial cell survival and mobilization of EPCs and HSCs (61). However, Ang-1 also suppresses angiogenesis in tumors and the heart (62,63). Ang-2, a natural antagonist of Ang-1, loosens the attachment of pericytes resulting in promoting sprouting angiogenesis in the presence of VEGF (54). However, when insufficient angiogenic signals are present, Ang-2 causes endothelial cell death and vessel regression (54).

3.3. Proteases

In the process of ECM remodeling during vessel sprouting, proteases such as urokinase plasminogen activator (uPA) and its inhibitor PAI-1, matrix metalloproteinases (MMPs) and tissue inhibitor of MMP, TIMPs, heparinases, and cathepsins play important roles (64,65). HSPGs present in the basement membranes sequester proangiogenic growth factors such as VEGF and bFGF. Proteinases liberate matrix-bound proangiogenic factors,

thus facilitating sprouting angiogenesis. MMP-9 and MMP-2 are known to be required for the mobilization of the sequestered VEGF and thus the initiation of tumor angiogenesis (66). Although MMP-9 does not effectively degrade perlecan or HSPGs, it degrades type IV collagen effectively, possibly disrupting the organization of basement membranes including HSPGs and perlecan, thus leading to the release of proangiogenic VEGF. Stromal and immune cells are known to produce MMPs (67). Following matrix degradation and the release of VEGF, angiogenesis is initiated and tumors begin to grow resulting in the further recruitment of immune cells and fibroblasts. The effect of MMP-9 in releasing matrix-bound VEGF is potentially important at the early stage of tumor progression associated with angiogenic switch, it is not likely, however, to be required after the angiogenesis has been initiated (68). Although insufficient breakdown of ECM prevents vascular endothelial cells from migrating into the interstitium, excessive degradation removes essential support and guidance for migrating endothelial cells, thus leading to sustained angiogenic response (64). Following degradation of basement membranes by MMPs, cryptic domains of partially degraded collagens with proangiogenic activity become exposed (69). On the other hand, proteinases are known to liberate matrix-bound or matrix comprising angiogenesis inhibitors such as endostatin (12), canstatin (16), arresten (15), tumstatin (17,70), restin (14), endorepellin (71), and thrombospondin (TSP)-1, resulting in the resolution of angiogenesis. Expression of MMPs increases as the tumors grow larger, and the levels of MMPs in serum and urine are known to be useful indicators of tumor progression and metastasis. Degradation of basement membranes by MMPs therefore acts as both a positive (at the early stage) and a negative (at the middle to late stage) regulator of tumor angiogenesis (68,72). Interestingly, a domain of MMP-2, PEX, can inhibit the interaction of MMP-2 and $\alpha\text{v}\beta 3$ integrin, thus leading to the suppression of tumor growth (73).

3.4. Ephrins

The Eph family of receptor tyrosine kinases and corresponding ligands ephrins had originally been identified to determine embryonic patterning and neuronal targeting (74). EphA receptors bind the ephrinA ligands; EphB receptors bind ephrinB ligands, with the exception of EphA4 binding to both ephrins (75). Mice deficient in ephrinB2 or EphB4 die during embryogenesis with severe cardiovascular defects, suggesting the involvement in the primary capillary network remodeling and patterning in embryonic vasculatures (76,77). Reciprocal expression pattern of ephrinB2 and EphB4 in arterial and venous endothelial cells, suggests that they might interact at the arterial-venous interface (78). EphA2 was observed in tumor-associated vascular endothelial cells, ephrinA1 was detected in tumor as well as endothelial cells and soluble EphA2-Fc exhibited anti-angiogenic and anti-tumor effect, suggesting the involvement in tumor angiogenesis (79).

3.5. Integrins

Integrins, cell surface receptors for extracellular matrix, exist as heterodimers of α and β subunits, forming at least 25 different combinations of receptors with distinct and overlapping specificity for ECM proteins (80). Integrins transmit bidirectional “outside-in” or “inside-out” signals (4). The $\alpha\text{v}\beta 3$ integrin is predominantly expressed on endothelial cells in tumor vasculature (81), and antibodies against $\alpha\text{v}\beta 3$ or $\alpha\text{v}\beta 5$ integrin

or RGD peptide recognized by these receptors efficiently blocked angiogenesis in tumors and retina (7,82,83). Antibodies against $\alpha v\beta 3$ interfered with bFGF-induced angiogenic effect, and antibodies against $\alpha v\beta 5$ interfered with VEGF-induced angiogenic effect (83). Genetically engineered mice lacking $\beta 3$ or $\beta 5$ -integrin are fertile and viable, and αv -integrin deficient mice showed extensive angiogenesis (8,84,85). Mice lacking one or both of these integrins ($\beta 3$ or $\beta 5$ -integrin) exhibited enhanced tumor growth and angiogenesis (85), suggesting the possibility that these integrins serve as negative regulators of angiogenesis. Previous reports have demonstrated the *trans*-dominant negative regulation of both the $\alpha 5\beta 1$ and $\alpha 2\beta 1$ integrin by $\alpha_{Iib}\beta 3$ integrin (86). Apparent discrepancy on the function of $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins might be mediated via *trans*-dominant inhibition of other proangiogenic integrins ($\alpha 5\beta 1$, $\alpha 1\beta 1$, or $\alpha 2\beta 1$ integrin) in endothelial cells (4). Thus, reagents directed at $\alpha v\beta 3$ or $\alpha v\beta 5$ integrin might indirectly inhibit proangiogenic function of other integrins such as $\alpha 5\beta 1$ integrin, instead of directly inhibiting proangiogenic activity of $\alpha v\beta 3$ or $\alpha v\beta 5$ integrin. Inhibitory action of $\alpha v\beta 3$ integrin on the level of Flk1, resulting in reduced responses to VEGF (87), further support the function of $\alpha v\beta 3$ integrin as a negative regulator of angiogenesis.

3.6. Cadherin

Vascular endothelial cadherin (VE-cadherin), a transmembrane glycoprotein located at adherent junctions of endothelial cells, plays a pivotal role in maintaining vascular integrity. Cytoplasmic tail of VE-cadherin interacts with β - and γ -catenin, which in turn promote the anchorage to actin cytoskeleton. Targeted inactivation or truncation of the VE-cadherin gene resulted in embryonic lethality resulting from altered vascular remodeling and impairment of VEGF-mediated endothelial cell survival and angiogenesis (88), suggesting that VE-cadherin is required for proper angiogenic process. In fact, antibodies against VE-cadherin blocked angiogenesis and tumor growth (89).

4. Mechanisms of Tumor Angiogenesis

Primary tumors and metastases initially exist as small avascular masses, and avascular *in situ* carcinomas may exist for months or years, consequently remaining as a small mass of a few cubic millimeters. Subsequently, some tumor cells switch to the angiogenic phenotype and induce the ingrowth of new capillary blood vessels to allow further tumor growth (10,90). Many primary epithelial tumors, originally separated from underlying blood vessels by basement membranes, develop in this manner, requiring the degradation of basement membranes to access the vasculatures. Expansion of tumor mass occurs not only by increased perfusion of blood through the tumor, but also by paracrine stimulation of growth factors and matrix proteins produced by the newly formed capillary endothelium (11). The switch to the angiogenic phenotype depends on a net balance of proangiogenic factors and antiangiogenic factors. Proangiogenic factors include bFGF, VEGF, and other factors secreted from tumor cells or infiltrating macrophages or mobilized from the ECM. Increased levels of proangiogenic factors accompanied by decreased levels of antiangiogenic factors are required for the 'angiogenic switch'.

In nonangiogenic *in situ* tumors or in dormant micrometastases, proliferation of tumor cells continues, but is balanced by high rates of apoptosis of tumor cells (91). The

angiogenic switch in human tumors are driven by: (1) angiogenic oncogenes which up-regulate proangiogenic factors and/or down-regulate antiangiogenic factors; (2) hypoxia leading to the activation of HIF-1 resulting in the activation of VEGF; (3) fibroblasts in the tumor bed producing proangiogenic factors; and (4) endothelial progenitor cells derived from bone marrow which migrate into tumor vasculatures (92). Once the angiogenic switch is turned on, neovessels converge on the dormant *in situ* tumors and tumor cells cluster around each microvessel. The angiogenic switch is associated with a marked decrease in tumor cell apoptosis.

The interaction of tumor cells with host blood vessels has been described in association with VEGF and Ang-2 (53); tumor cells initially home in on and grow by co-opting existing blood vessels, and start off as well-vascularized small tumors (93). The host blood vessels regress in response to the inappropriate co-option as a result of elevated autocrine expression of Ang-2, starve the tumor, and render tumors avascular and hypoxic. The co-opted blood vessels undergo apoptosis and the tumor becomes avascular and hypoxic, leading to marked induction of VEGF from tumor cells. In the presence of elevated levels of VEGF, regression of destabilized co-opted blood vessels will stop, and subsequent sprouting angiogenesis from these blood vessels will allow for the survival and growth of the tumor. The microenvironment with high levels of Ang-2 may explain why tumor vasculatures fail to mature, and tend to be leaky and hemorrhagic (53).

Antiangiogenic therapy can inhibit endothelial cell proliferation in a tumor bed or increase endothelial cell apoptosis. Endothelial cells in the tumor bed have significantly higher proliferation rates than quiescent endothelium and they should be more susceptible to cytotoxic agents. Several chemotherapeutic agents, in fact, inhibit angiogenesis in addition to inducing tumor cell death. For example, paclitaxel, which inhibits microtubule polymerization, inhibits proliferation and migration of endothelial cells, and inhibits tumor growth (94). Continuous administration of chemotherapeutic agents such as cyclophosphamide or vinblastine at a lower dosage than the usual maximum tolerated dose (antiangiogenic schedule) resulted in complete tumor regression and long-term survival in experimental animal models (95).

5. Inhibitors of Angiogenesis (see Table 3)

5.1. Endostatin

Type XVIII collagen is a triple helical molecule harboring several heparan sulfate side-chains, thus classified as a major proteoglycans of epithelial and vascular basement membranes (96,97). The C-terminal globular noncollagenous (NC) domain contains the antiangiogenic fragment, endostatin (12). This antiangiogenic domain is separated from an upstream trimerization region by a protease-sensitive hinge (96). Upon cleavage within this hinge region, endostatin will be released into circulation and tissues (98). Type XVIII is known to be involved in the normal development of vasculature in the retina, and a splice mutation in human type XVIII collagen has been associated with Knobloch syndrome, a disease with insufficient development of retinal vasculatures leading to retinal degeneration and blindness (99).

Type XV collagen, sharing homology with type XVIII collagen (100), is a disulfide-bonded proteoglycan containing chondroitin sulfate side-chains. These collagens are

Table 3
Endogenous Basement Membrane Derived Angiogenesis Inhibitors

Angiogenesis inhibitor	Parent protein	Domain	Receptors	Inhibitory activities on EC
Endostatin	$\alpha 1$ chain of type XVIII collagen	NC1	Glypicans, flk-1, $\alpha 5\beta 1$ -integrin	Proliferation, migration, tube formation, survival, tumor growth
Restin	$\alpha 1$ chain of type XV collagen	NC10	Unknown	Proliferation, migration, tumor growth
Tumstatin	$\alpha 3$ chain of type IV collagen	NC1	$\alpha v\beta 3$ -integrin	Proliferation, tube formation, survival, protein translation, tumor growth
Arresten	$\alpha 1$ chain of type IV collagen	NC1	$\alpha 1\beta 1$ -integrin	Proliferation, migration, tube formation, survival, tumor growth
Canstatin	$\alpha 2$ chain of type IV collagen	NC1	$\alpha 3\beta 1$ -integrin	Proliferation, migration, tube formation, survival, tumor growth
$\alpha 6(IV)NC1$	$\alpha 6$ chain of type IV collagen	NC1	Unknown	Proliferation
Endorepellin	Perlecan	Domain V	$\alpha 2\beta 1$ -integrin	Migration, tube formation, survival, blood vessel growth <i>in vivo</i>

multiplexin subclass of nonfibrillar collagens containing an N-terminal NC domain, triple-helical regions with multiple interruptions by NC domains, and C-terminal NC1. Type XV collagen is widely distributed in several basement membranes of various tissues, including vascular basement membranes (101). Studies from mice deficient in type XV collagen revealed the important structural role of this collagen in stabilizing skeletal muscle cells and microvessels (102). Similar to type XVIII collagen, the C-terminal NC domain, termed as restin, was shown to possess anti-angiogenic activity (14,103).

Endostatin, a 20 kDa C-terminal NC1 domain of type XVIII collagen, possesses potent antiangiogenic activity (12). Endostatin was first isolated from the conditioned medium of murine hemangioendothelioma cells. Endostatin inhibits endothelial cell proliferation, migration and tube formation in vitro (104–106) and possesses potent inhibitory effect on tumor growth in vivo (12,107). The inhibitory effects of endostatin on the expression of VEGF in tumor cells and on vascular permeability had been reported (108). Endostatin also blocks VEGF-mediated proangiogenic signaling via direct interaction with flk-1 (109). Recent reports have demonstrated the therapeutic potential of endostatin in nonneoplastic disorders with involvement of angiogenic process such as rheumatoid arthritis and proliferative diabetic retinopathy (110,111). These reports have also demonstrated the antiangiogenic activity of synthetic peptide derived from N-terminal domain of human endostatin in vitro and in vivo (112).

Cell surface receptors for endostatin had been extensively studied recently. Rehn et al. reported that recombinant human endostatin interacted with $\alpha 5$ - and αv -integrins on the surface of human endothelial cells, and demonstrated the functional significance of this interaction (113). More recently, interaction of endostatin with $\alpha 5\beta 1$ integrin in association with cell surface HSPGs and caveolin have been reported (114,115). The interaction of endostatin with $\alpha 5\beta 1$ integrin led to the activation of caveolin-associated Src tyrosine kinase, p190RhoGAP phosphorylation, followed by inactivation of RhoA, and disassembly of actin stress fibers and focal adhesion, the mechanism potentially involved in the inhibitory effect of endostatin on endothelial cell migration (115). Sudhakar et al. reported that human endostatin binding to $\alpha 5\beta 1$ integrin led to the inhibition of the activation of focal adhesion kinase and subsequent inhibition of mitogen-activated protein kinases (MAPKs) including extracellular signal-regulated kinase (ERK)-1/2 and p38MAPK, resulting in the inhibition of endothelial cell migration (116). In addition, heparan sulfate glycosaminoglycans, or glypicans, were reported to bind to endostatin, and this interaction was important for mediating endostatin's antiangiogenic activities in vitro (117). The requirement of E-selectin, an inducible leukocyte adhesion molecule specifically expressed on endothelial cells, for the antiangiogenic activity of endostatin has also been reported (118). Suppressive effect of endostatin on the expression of Id1 and Id3, essential proteins in regulating cell growth and differentiation, and the transcription factor Ets-1 which promotes angiogenesis via inducing several target genes such as MMP-1, MMP-3, MMP-9 and $\beta 3$ integrin, had been recently reported (119).

5.2. Tumstatin

Type IV collagen, the most abundant constituent of the basement membranes, is expressed as six distinct α -chains, namely, $\alpha 1$ – $\alpha 6$ (120), assembles into triple helices,

and forms organized networks. Network assembly of type IV collagen is essential for structural integrity and biological functions of basement membranes. In general, type IV collagen possesses the ability to promote cell adhesion, migration, differentiation, and cell growth (121). Although type IV collagen is found only in basement membranes in normal conditions, it is associated with organ fibrosis and accumulates in tumor interstitium. The α -chains consist of N-terminal cysteine-rich 7S domain, the middle collagenous triple helical domain with Gly-X-Y repeats interrupted by short NC sequences, and a C-terminal globular NC1 (2). Superstructures self-associate from triple helical monomers to form either dimers (via interactions of NC1 domains) or tetramers (via interaction of 7S domains), with 56 possible combinations (122). The $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains are ubiquitously distributed in human basement membranes and heterotrimers composed of 2 $\alpha 1(\text{IV})$ and 1 $\alpha 2(\text{IV})$ chains are predominant (123). In contrast, the localization of the other four isoforms is tissue- and organ-specific (124,125). The distribution of the $\alpha 3(\text{IV})$ chain is limited to certain basement membranes, such as glomerular basement membrane, several basement membranes of the cochlea, ocular basement membrane of the anterior lens capsule, Descemet's membrane, ovarian and testicular basement membrane (126), and alveolar capillary basement membrane (125,127). This chain is absent from epidermal basement membranes of the skin and the vascular basement membrane of liver (125).

The NC1 domain of type IV collagen is considered to play a crucial role in the assembly of type IV collagen to form trimers, and thus influence basement membrane organization and modulation of cell behavior (2,121). Petitclerc et al. demonstrated the potent biological activity of recombinant $\alpha 2(\text{IV})$, $\alpha 3(\text{IV})$, and $\alpha 6(\text{IV})$ NC1 domains to inhibit angiogenesis and tumor growth (18).

We identified the pivotal role of recombinant human $\alpha 3(\text{IV})\text{NC1}$ in inhibiting the proliferation of capillary endothelial cells and blood vessel tube formation, and also in inducing endothelial cell specific apoptosis (17). We named this domain as "umstatin" (for its unique property of causing "tumor-stasis"), to add another member to the family of endogenous inhibitors of angiogenesis derived from larger proteins, such as angiostatin, endostatin and restin (12–14). Tumstatin further inhibited in vivo neovascularization in matrigel plug assays and suppressed tumor growth of human renal cell carcinoma (786-O) and prostate carcinoma (PC-3) in xenograft mice models (17). Endothelial cell apoptosis of tumor vasculatures was induced in tumstatin-treated mice with PC-3 tumors.

Goodpasture syndrome is an autoimmune disease characterized by pulmonary hemorrhage and/or rapidly progressive glomerulonephritis (127). These symptoms are caused by the disruption of glomerular and pulmonary alveolar basement membrane through immune injury associated with autoantibody against $\alpha 3(\text{IV})$ NC1 (127). The most probable disease-related pathogenic autoepitope was identified in the N-terminal portion (128,129) and was further confined to be within the N-terminal 40 amino acids (130,131). Truncated tumstatin (tum-1) lacking N-terminal 53 amino acids encompassing the pathogenic Goodpasture autoepitopes, was fully active in inhibiting tumor growth and angiogenesis, and another deletion mutant tum-2, consisting of 124 amino acids in the N-terminal half portion of tumstatin possessed equivalent antiangiogenic activity specific to endothelial cells (17). In contrast, deletion mutant tum-3 consisting of 120 amino acids in the C-terminal half portion of tumstatin, failed to inhibit

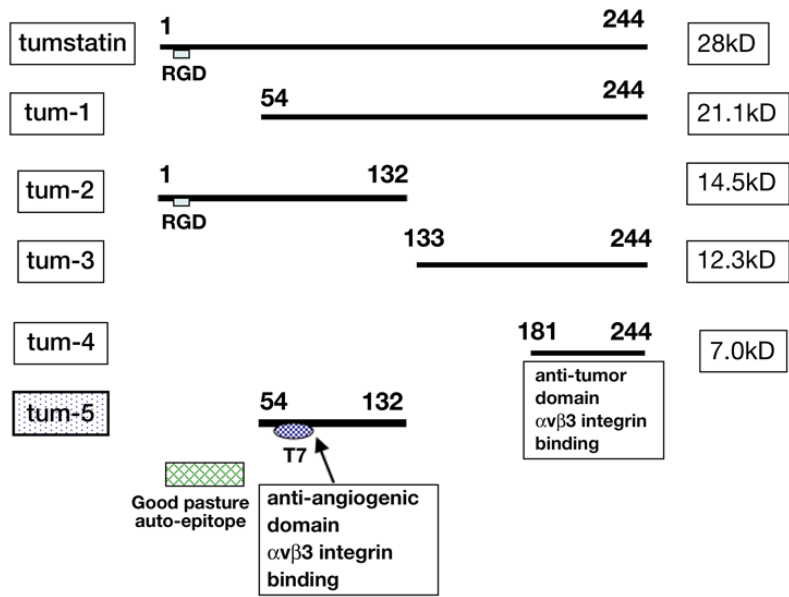


Fig. 2. Anti-angiogenic domain of tumstatin. Tumstatin, a 244-kDa domain derived from C-terminal NC1 domain of $\alpha 3$ chain of type IV collagen, possesses antiangiogenic tum-5 domain that binds to $\alpha v\beta 3$ integrin in an RGD-independent manner. This domain does not include Goodpasture autoepitope and the core antiangiogenic domain is located at T7 peptide domain. C-terminal tum-4 domain possesses antitumor activity with binding to $\alpha v\beta 3$ integrin in an RGD-independent manner.

angiogenic responses in endothelial cells. These results collectively suggested the localization of antiangiogenic domain within the amino acids 54–124 of tumstatin, which differs from the Goodpasture auto-epitope (*see* Fig. 2).

Previously, the $\alpha 3(\text{IV})\text{NC1}$ domain had been shown to bind and inhibit the growth of melanoma cell lines *in vitro* via amino acids 185–203 of $\alpha 3(\text{IV})\text{NC1}$ domain (132). Deletion mutant tum-4 consists of 64 amino acids in the C-terminus of tumstatin, which includes the 185–203 peptide region. Although tum-4 did not inhibit the proliferation of endothelial cells, it inhibited the proliferation of melanoma cells (WM-164). The antitumor activities of tumstatin were thus considered to localize in distinct region of tumstatin, different from antiangiogenic domain (17).

The NC1 domain of $\alpha 1$ and $\alpha 2$ chain of type IV collagen (arresten and canstatin, respectively) also possesses antiangiogenic activity. Arresten, the 26-kDa NC1 domain of the $\alpha 1$ chain of type IV collagen, inhibits endothelial cell proliferation, migration, tube formation, and *in vivo* neovascularization using matrigel plug assay. Arresten also inhibits the growth of two human xenograft tumor models in nude mice and the development of tumor metastases. The antiangiogenic activity of arresten was mediated via interaction with cell surface proteoglycans and the $\alpha 1\beta 1$ integrin on endothelial cells (15). Canstatin, an endogenous 24-kDa fragment of human $\alpha 2$ chain of type IV collagen inhibits endothelial cell proliferation, migration and murine endothelial cell tube formation. In addition, canstatin potently induced apoptosis of endothelial cells, without exhibiting any effects on nonendothelial cells. Treatment with canstatin did not

inhibit the activation of ERK1/2. The proapoptotic effect of canstatin on endothelial cells was associated with a down-regulation of the antiapoptotic protein, FLIP. Canstatin also suppressed in vivo growth of tumors in two human xenograft mouse models with decreased CD31-positive tumor vasculatures (16). The cell surface receptors of canstatin are considered as $\alpha v\beta 3$ and $\alpha 3\beta 1$ integrin. Canstatin inhibited the activation of PI3-kinase/Akt resulting in the induction of apoptosis dependent upon signaling events transduced through Fas membrane death receptors (133).

5.2.1. Tumstatin Binds to $\alpha v\beta 3$ Integrin

Within the N-terminal portion of tumstatin, there is a RGD sequence (amino acids 7–9). In general, the RGD sequence is considered as an important binding site for $\alpha v\beta 3$ integrin. Previous studies have identified the 185–203 amino acid sequence of $\alpha 3(IV)NC1$ as a ligand for $\alpha v\beta 3$ integrin and responsible for the associated antitumor cell property (134). We found distinct additional RGD independent $\alpha v\beta 3$ integrin binding site within 54–132 amino acids of tumstatin (135). This site does not include the RGD sequence, and, as described above, this site is not essential for inhibition of tumor cell proliferation but necessary for the antiangiogenic activity. A fragment of tumstatin containing 54–132 amino acids could bind both endothelial cells and melanoma cells, but could only inhibit proliferation of endothelial cells, with no effect on tumor cell proliferation. Another fragment of tumstatin containing the 185–203 amino acid (tum-4) could bind both endothelial cells and melanoma cells, but could only inhibit the proliferation of melanoma cells. These results may suggest the involvement of additional cell-specific ligand receptor interactions for exerting cell-specific antiproliferative effects. The presence of cyclic RGD peptides did not affect the $\alpha v\beta 3$ integrin-mediated antiangiogenic activity of tumstatin, although significant inhibition of endothelial cell binding to vitronectin was observed. Thus, the two distinct RGD-independent $\alpha v\beta 3$ binding sites on tumstatin mediated two separate antitumor activities, suggesting unique $\alpha v\beta 3$ integrin mediated mechanisms governing the two distinct anti-tumor properties of tumstatin (135).

5.2.2. Tum-5 Domain

In order to directly demonstrate the antiangiogenic activity of the putative 54–132 amino acids of tumstatin (tum-5 domain), the recombinant tum-5 was produced in *Escherichia coli* and *Pichia pastoris*. Recombinant tum-5 proteins were not recognized by antisera obtained from patients with Goodpasture syndrome, excluding the possibility that these recombinant proteins might induce an autoimmune disorder. Tum-5 specifically inhibited proliferation and caused apoptosis of endothelial cells with no significant effect on nonendothelial cells such as melanoma cell line (WM-164) or prostate carcinoma cell line (PC-3) (136). Tum-5 also inhibited tube formation of endothelial cells on matrigel and induced G1 cell cycle arrest of endothelial cells. In addition, antiangiogenic effect of tum-5 was demonstrated in vivo using both a Matrigel plug assay in C57BL/6 mice, and human prostate cancer (PC-3) xenografts in nude mice. Tum-5 at 1 mg/kg significantly inhibited growth of PC-3 tumors in association with a decrease in CD31 positive tumor vasculatures. Cell attachment assays revealed that tum-5 could bind to $\alpha v\beta 3$ integrin in an RGD-independent manner, and was thus independent of vitronectin binding. Competition proliferation assays utilizing soluble $\alpha v\beta 3$

integrin protein to compete $\alpha\beta3$ integrin receptors on the cell surface revealed that the antiproliferative effect of tum-5 was reversed dose-dependently with an increasing amount of $\alpha\beta3$ soluble protein, suggesting the requirement of tum-5- $\alpha\beta3$ integrin interaction in antiangiogenic effect of tum-5 (136). These results suggested the potential role of $\alpha\beta3$ integrin in negative regulation of angiogenesis by binding to tum-5.

5.2.3. Tumstatin Peptide

Reduction and alkylation of tumstatin and tum-5 failed to alter the antiangiogenic activity in vitro and in vivo, suggesting this activity was independent of disulfide bond requirement (137). Therefore, five overlapping synthetic peptides were designed so that they would cover the tum-5 domain. Among these peptides, only the T3 (69–88 amino acids) and T7 peptide (74–98 amino acids) inhibited proliferation and induced apoptosis specifically in endothelial cells. T3 peptide, similar to tumstatin and the tum-5 domain, could bind and exert antiangiogenic effects via the $\alpha\beta3$ integrin in an RGD-independent manner. Restoration of a disulfide bond between two cystines within the T3 peptide did not affect on the antiangiogenic activity of T3 peptide. Antiangiogenic effect of the peptides was further confirmed in vivo using a Matrigel plug assay in C57BL/6 mice. These results suggested that the antiangiogenic activity of tumstatin was localized to a 25 amino acid region of tumstatin (T7 peptide) (137).

5.2.4. Tumstatin Inhibits Protein Synthesis in Endothelial Cells

Apoptosis, programmed cell death, is regulated in part at the level of protein synthesis, and is generally associated with inhibition of cap-dependent protein translation (138–140). Because tumstatin selectively stimulates apoptosis of endothelial cells via binding to $\alpha\beta3$ integrin, the potential effects of tumstatin peptides on protein synthesis have been examined. Tum-5, T3, and T7 peptide inhibited protein synthesis in endothelial cells, but not in other nonendothelial cells. In contrast, endostatin did not exhibit similar effects on protein synthesis. Tumstatin peptides exhibited endothelial cell-specific inhibitory effect on cap-dependent translation, and this inhibitory effect was not observed in $\beta3$ -integrin-deficient endothelial cells (141).

In many cell types, ligand binding to integrin induced phosphorylation of focal adhesion kinase (FAK) and subsequent activation of various signaling molecules (142,143). For instance, phosphorylated FAK interacts with and activates phosphatidylinositol 3-kinase (PI3-kinase) and protein kinase B (PKB/Akt), leading to cell survival (142,144). In fact, inhibition of PI3-kinase in endothelial cells has been shown to repress protein synthesis (145). Tumstatin peptides inhibited phosphorylation and activation of PI3-kinase and phosphorylation of Akt in endothelial cells (141). Akt activates Rapamycin/FKBP-target 1 protein (RAFT1), also known as mammalian target of rapamycin (mTOR), which in turn phosphorylates eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP1) (146,147). Unphosphorylated 4E-BP1 forms complex with eIF4E and inhibits cap-dependent translation (148). In fact, stimulation of cells with growth factors induces phosphorylation of 4E-BP1, dissociation of 4E-BP1 from eIF4E, leading to the induction of protein translation (147,148). Tumstatin peptides suppressed mTOR kinase activity, phosphorylation of 4E-BP1, and dissociation of 4E-BP1 from eIF4E, thus resulting in inhibition of cap-dependent translation. In contrast, tumstatin peptides failed to inhibit phosphorylation of ERK1/2 upon vitronectin

attachment or stimulation with VEGF in endothelial cells. Overexpression of constitutive active Akt resulted in reversal of tumstatin peptide-induced inhibition of cap-dependent translation. These results suggested the possible inhibitory effects of tumstatin peptides on protein synthesis of endothelial cells through negative regulation of mTOR signaling. Alternatively, interaction of tumstatin peptide with $\alpha v\beta 3$ integrin may induce negative regulatory signals counteracting growth factor-initiated cell survival signals (see Fig. 3).

5.2.5. Endogenous Tumstatin Inhibits Angiogenesis and Tumor Growth

In addition to the antiangiogenic effect of exogenously added tumstatin, the endogenous functions of tumstatin have been reported by Hamano et al. (70). Mice deficient in $\alpha 3$ chain of type IV collagen, a precursor of tumstatin, exhibited normal pregnancy, development, and wound healing process, but had accelerated pathological angiogenesis and tumor growth. Although tumstatin inhibited angiogenesis in mice with the physiological expression levels of $\beta 3$ integrin, inhibitory effect of tumstatin was not observed in $\beta 3$ integrin-null mice, suggesting the requirement of $\beta 3$ integrin in tumstatin's antiangiogenic effect (70). Treatment of basement membranes with MMP-9 generated tumstatin fragments in vitro. Other MMPs—MMP-2, 3, and 13—could release tumstatin fragment from basement membranes, although the efficiency was significantly less than MMP-9. In fact, mice deficient in MMP-9 exhibit significantly decreased circulating blood concentrations of tumstatin compared with wild-type mice. The growth of Lewis lung carcinoma tumors was similar in both MMP-9(+/+) and MMP-9(-/-) mice until the tumors reached 500 mm³, but the tumors on MMP-9(-/-) mice exhibited accelerated tumor growth afterwards. Intravenous administration of tumstatin to raise the concentration of tumstatin to normal levels resulted in the retardation of tumor growth similar to the level of wild-type mice, suggesting the importance of physiological levels of tumstatin in regulating tumor growth (70). MMP-9 has been implicated as a positive regulator of angiogenic switch, leading to reduced tumor growth and tumor vasculatures upon genetic ablation of MMP-9 in the early stage of tumor growth (66). However, pharmacological inhibition of MMP-9 resulted in acceleration of tumor growth (72). Whereas MMP-9 may mediate the angiogenic switch leading to the initial burst of tumor growth, it also suppresses tumor growth by generating endogenous inhibitors of angiogenesis such as tumstatin (70). The physiological endogenous anti-angiogenic role of TSP-1 and endostatin via binding to CD36 and $\alpha 5\beta 1$ integrin, respectively, had also been recently demonstrated (149).

5.2.6. Application of Tumstatin Peptide on Non-neoplastic Disorder

Diabetic nephropathy is complicated in 30–40% of patients with type 2 diabetes and is the most common pathological disorders predisposing end-stage renal diseases (ESRD) in Japan and in the Western World (150). Hyperglycemia is involved in the progression of diabetic nephropathy and early alterations in diabetic nephropathy include glomerular hyperfiltration, glomerular and tubular epithelial hypertrophy, and the development of microalbuminuria (151). These early alterations are followed by the development of glomerular basement membrane thickening, the accumulation of extracellular matrix components in the mesangium as well as in the interstitium, and the increase of urinary albumin excretion, eventually leading to glomerulosclerosis and

5.3. Endorepelin

5.3.1. Perlecan

Perlecan is a major HSPG of basement membranes and vascular and avascular ECM, involved in regulating cell growth, differentiation, cell adhesion, and the development of blood vessels, cartilage, and the nervous system (155). Perlecan-null mutations result in early embryonic lethality accompanied by severe cephalic and cartilage abnormalities (156). Embryos that survive initially usually develop later malformations of the cardiovascular system (157). In humans, two rare skeletal disorders, dyssegmental dysplasia silver-handmaker type (DSSH) and Schwartz-Jampel syndrome (SJS) are caused by mutations of genes encoding perlecan (158,159). Perlecan is considered to exert proangiogenic effects, because it binds to and protect growth factors from degradation and it interacts with adhesion molecules (160).

5.3.2. Endorepellin, an Antiangiogenic C-Terminal Fragment of Perlecan

Perlecan, a C-terminal 85-kDa domain V of perlecan inhibits endothelial cell migration, tube formation, and growth of blood vessels (71). Endorepellin is suspected of inhibiting angiogenesis in a dominant-negative fashion, endothelial cells secrete perlecan. Endorepellin reversibly alters the actin cytoskeleton of endothelial cells, potentially associated with its effects on cell motility and other alterations in cell morphology (161). The interaction of endorepellin with $\alpha 2\beta 1$ integrin triggers a unique signaling pathway that causes an increase in the second messenger camp; activation of protein kinase A and FAK; transient activation of p38 mitogen-activated protein kinase and heat shock protein 27, followed by a rapid down-regulation of the latter two proteins; and ultimately disassembly of actin stress fibers and focal adhesions associated with the inhibitory effect on endothelial cell migration and angiogenesis (161). Collectively, a family of C-terminal fragment of endogenous matrix proteins (endostatin, tumstatin and endorepellin) harbors potent antiangiogenic effects potentially generated via processing by proteases.

5.4. Anti-VEGF Reagents

Based on the well-known role of VEGF in promoting angiogenesis and tumor growth, therapeutic strategies to block the biological effect of VEGF had been developed and, to date, clinically tested on patients with refractory tumors. The therapeutic effect of neutralizing anti-VEGF or VEGF receptor antibody, small molecule inhibitor of tyrosine kinase receptor of VEGF in experimental tumor models has been reported (79).

Observation of proteinuria upon treatment with anti-VEGF antibody or soluble flt-1 (an antagonist of VEGF) has been reported in experimental animal models (162) and clinical trials, suggesting the possible side effect on glomerular filtration barrier.

6. Conclusion

Here, we described on the biological roles of angiogenic response in developing tumors and the mediators and inhibitors of angiogenesis, emphasizing the endogenous inhibitors of angiogenesis derived from basement membrane proteins. Further publications on the role of angiogenesis in developing cancer implicate the significance of this research field and its potential to lead to the development of novel therapeutic approaches to halt and regress tumor growth. Investigation on the biological function of endogenous angiogenesis

inhibitors may provide insight into the regulatory mechanism of endothelial cells and other vascular cell components in the setting of various pathological disorders.

Acknowledgments

A portion of this study was supported by research grant from a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (YM), Grant-in-Aid from the Tokyo Biochemical Research Foundation (YM), and from the Japan Diabetes Foundation (YM). YM is a recipient of the 2001 Research Award from the Okayama Medical Foundation, the 2002 Research Award from the KANAE Foundation for Life & Socio-Medical Science, the 2002 Young Investigator Award from the Japan Society of Cardiovascular Endocrinology and Metabolism, the 2003 Research Award from the Kobayashi Magobei Memorial Foundation for Medical Science, the 2003 Research Award from the Ryobi Teien Memorial Foundation, the 2004 Research award from the Inamori Foundation, the 2004 Research award and from the SUZUKEN memorial foundation, the 2004 Research Award from the Sanyo Broadcast Academic and Cultural Foundation, and the 2005 Oshima Award (Young Investigator Award) from the Japanese Society of Nephrology.

I appreciate Dr. Raghu Kalluri (Beth Israel Deaconess Medical Center and Harvard Medical School, Boston) and Dr. Hirofumi Makino (Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan) for their mentorship in my research career.

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Metastasis

The Evasion of Apoptosis

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Summary

Metastasis, the spread of tumor cells from the primary site of growth to a secondary site, is the leading cause of cancer related deaths. The process of metastasis can be conceptually broken down into a series of sequential steps: acquisition of an invasive phenotype, intravasation, travel through the circulatory system, arrest at a secondary site, and extravasation and colonization at that site. The metastatic cell must be able to escape cell death at several steps in this process. Thus, it is not surprising that metastatic cells can be molecularly characterized by altered expression of several apoptosis genes and altered signaling through cell survival and death pathways. In addition, this same acquired resistance to cell death that has allowed metastatic cells to survive and colonize in a secondary organ has also permitted resistance to courses of chemotherapy. Herein, we describe the aberrant apoptotic signaling that metastatic cells have evolved, how this aberrant signaling impedes traditional chemotherapeutic approaches that induce cell death, and potential therapeutic approaches to overcome apoptotic resistance of metastasis.

Key Words: Metastasis; apoptosis; Bcl-2 family; TNF- α family; DAP kinase; maspin; transcription factors; anoikis; molecularly targeted therapy; chemotherapeutic resistance.

1. Introduction to Metastasis

Tumor metastasis is a significant contributor to death in cancer patients. More than 90% of cancer deaths are caused by the development of metastases, because the primary tumor can often be treated successfully if detected early (*1*). Simply defined, metastasis is the spread of malignant tumor cells from the primary tumor site to a secondary site within the body.

Metastasis is a complex, multistep process that can be divided into five stages as illustrated in *Fig. 1*. In the first stage, the primary tumor cells acquire an invasive phenotype. They exhibit alterations in cell-to-cell interactions, as well as cell-to-extracellular matrix (ECM) interactions leading to initiation of motility. The second stage, termed intravasation, culminates in the escape of tumor cells from the primary site and penetration into circulation, possibly via the lymphatic vessels. During the third stage, malignant cells passively and efficiently travel through the circulation until they reach a capillary bed and either adhere to the vessel walls or arrest as a result of size constraints. Extravasation is the fourth step of the metastatic process wherein tumor cells exit the circulatory vessels at the destined site. Following extravasation, metastatic tumor cells proliferate, forming micro-metastatic or macrometastatic lesions within the organ in a process called colonization.

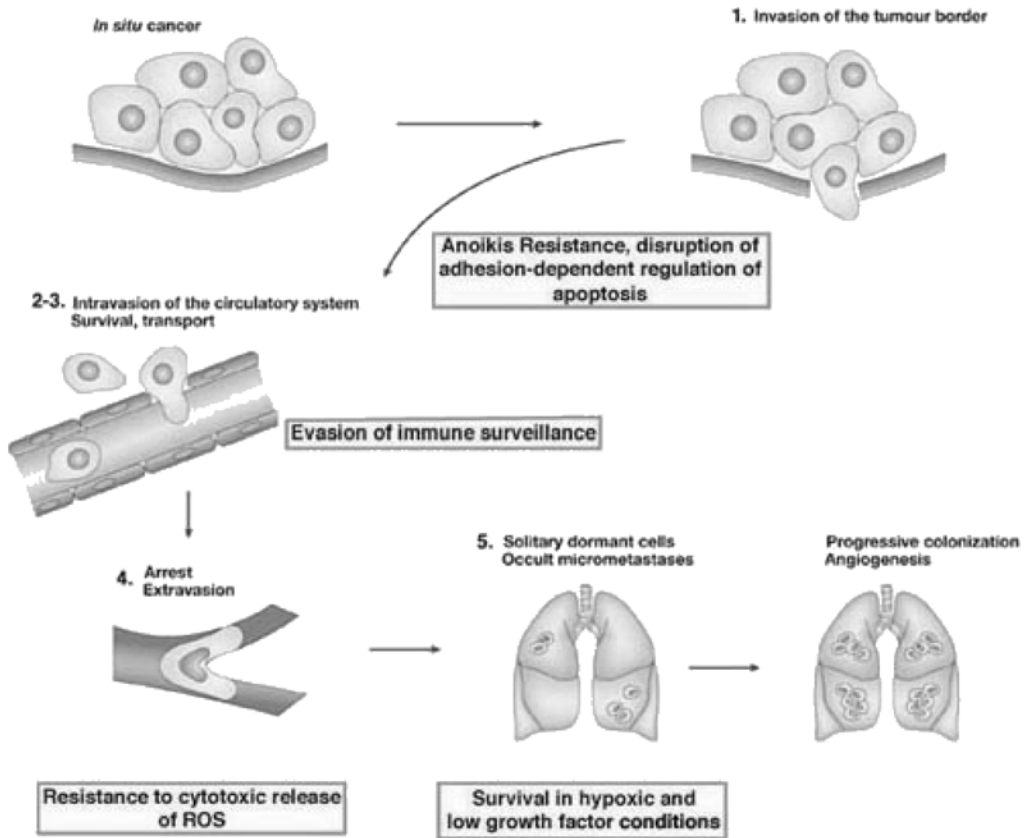


Fig. 1. Metastasis formation through evasion of apoptosis. The stages of metastasis (1–5) are shown: (1) Destruction and invasion of the basement membrane by *in situ* cancer through changes in cell–cell and cell–ECM interaction, and initiation of motility. (2–3) Intravasation, survival and transport of the metastatic cells via the lymphatic or the circulatory system. (4) Arrest in capillary bed and extravasation into the destined organ. (5) Proliferation of metastatic cells at the new location and colonization. Boxed text highlights apoptotic evasion events within each of these stages.

Metastasis is inherently an inefficient process (2). The initial steps of metastasis are performed with very high efficiency, whereas the final stage—initiation of colonization and persistence—is considerably less efficient. Elegant *in vivo* microscopy studies following the fate of malignant cells through the metastatic process have established that most cells die during extravasation and colonization (3). It is estimated that only 0.01 to 0.1% of malignant cells in circulation actually give rise to macrometastases (3). This inefficiency is caused primarily by cell death as a result of the many stresses that are introduced before and after the cells reach the new environment. Such stresses include the loss of cell–cell contact, recognition and destruction of the tumor cells by the immune system, and lack of necessary growth factors, all of which trigger apoptosis (4).

2. Apoptotic Resistance in Metastatic Progression

Apoptosis is a genetically determined process by which a cell self-destructs and commits suicide in order to maintain cellular homeostasis. It is hypothesized to block metastatic dissemination by eliminating misplaced cells. The process of apoptosis is the primary

contributor to metastatic inefficiency. The success of the metastatic process relies on the ability of malignant cells to evade apoptosis. A strong correlation between the metastatic aggressiveness of cell lines and resistance to apoptosis has been established from countless studies. In addition, there is evidence correlating disease prognosis and the apoptotic index, a measure of the amount of programmed cell death within tumor tissue specimens (5–11).

Acquired apoptotic resistance is critical at several steps in the metastatic cascade, but perhaps the most important stage is resistance to cell death induced by loss of cell–cell and cell–ECM contact. Matrix-independent survival of metastatic cancer cells before intravasation and during the passage through blood and/or lymph compartments is an essential component of the metastatic cascade. Detachment of cells from the ECM results in a type of programmed cell death referred to as anoikis. Some of the earliest evidence of anoikis resistance in metastatic cells was reported by Glinsky et al., who showed that higher survival of metastatic cancer cells compared with nonmetastatic or poorly metastatic cells was associated with the loss of homotypic and heterotypic aggregation and disruption of adhesion-dependent regulation of apoptosis (12). Since then, there have been numerous reports of anoikis resistance in metastatic cell lines and lesions. The molecular basis of this resistance will be discussed later in this chapter.

En route to metastatic colonization, malignant cells must also evade immune surveillance in circulation and at the site of colonization. They must develop a mechanism to escape cell death induced by cytotoxic lymphocytes and natural killer (NK) cells that recognize and destroy misplaced cells. Further, malignant cells must escape cytotoxicity induced by reactive oxygen species (ROS) release from endothelial cells while crossing the blood/tissue barrier during the process of extravasation (13). Finally, malignant cells must be able to successfully colonize at the secondary site. They must conquer hypoxic conditions and proliferate in an environment lacking the necessary growth factors.

It has been shown that even after successful formation of micrometastases, macroscopic tumors may not develop because of dormancy (3). Folkman and colleagues first described metastatic dormancy as the process in which tumor cells are metabolically active, but the rate of proliferation is balanced by the rate of apoptosis such that no net tumor growth occurs (14). A second type of dormancy involves significant numbers of nondividing solitary tumor cells that persist for long periods of time. These cells do not proliferate, as measured by retention of fluorescent nanospheres (these would be diluted upon cell division), nor do they undergo apoptosis (15). The nature of dormancy remains elusive, but genetic and/or environmental influences may shunt cells out of dormancy into an antiapoptotic, highly proliferative state allowing metastatic colonization to ensue.

As we shall see in this chapter, the process of apoptotic evasion is a genetically and molecularly regulated process. It not only creates a permissive environment for metastasis, but it also allows tumor cells to withstand the action of therapy (16).

3. Metastasis Assays

To discern the effects of apoptotic mediators on metastasis, an understanding of standard metastasis assays is in order. Measurements of metastasis discussed in this chapter range from *in vitro* and *in vivo* metastasis assays to clinical correlates in human tumor cohorts. Hallmarks of metastasis may be measured by cell culture-based assays such as soft agar colonization, wound scratch assays, and Boyden chamber chemotaxis assays, which assess anchorage-independent growth, motility, and invasion. The process of metastasis can only truly be assayed *in vivo* with mouse modeling experiments. There are

genetically engineered models of metastasis (transgenics) as well as transplantable murine models—syngeneic (host and tumor cells derived from similar genetic background) and human–mouse xenograft (host must be immunocompromised to prevent tissue rejection) (reviewed in ref. 17). Both syngeneic and xenograft models can be employed in either of the two classical metastasis assays—experimental and spontaneous. In the experimental metastasis assay, tumor cells are directly injected into the circulation, commonly via the tail vein, portal vein, or arterial circulation (intracardiac). A significant outcome of the experimental metastasis assay has been generation of clonal variants with differing metastatic potential, resulting from successive injections and explants (17). However, in the experimental metastasis assay, early steps of the metastatic process, invasion and intravasation, are bypassed. In the spontaneous metastasis assay, orthotopic transplantation (injection of tumor cells into the tissue from which the tumor was derived) can lead to spontaneous metastasis to distant sites, which may more accurately reflect the metastatic process in its entirety. Strategies to image *in vivo* metastases typically include bioluminescence (luciferase or green fluorescent protein-labeled tumor cells), magnetic resonance imaging, or positron enhanced tomography (17).

Human tumor cohort studies are by far the most relevant to human disease, but provide merely indirect, correlative information. Immunohistochemistry (IHC), reverse-transcription (RT)-PCR, and immunoblotting analysis are employed to measure gene and protein expression in tumor cohorts. This expression data can then be correlated to a number of clinicopathological factors including tumor grade, stage, survival, and risk for local or distant metastases. The significance of these associations thus dictates whether the protein of interest is a reliable prognostic indicator.

4. Molecular Mechanisms of Apoptotic Resistance

Tumor cells go through multiple molecular and genetic changes en route to achieving a metastatic state. Colonization at a secondary site requires the acquisition of an antiapoptotic, prosurvival phenotype. Apoptotic resistance may be achieved through one or more of the following general mechanisms:

1. Disruption of caspase function by mutation of caspase genes, altered expression of inhibitors of apoptosis (IAPs) and/or death-associated protein kinases (DAP kinases).
2. Altered activity and/or expression of components of the intrinsic/mitochondrial pathway of apoptosis, which includes Bcl-2 family members, Apaf-1 and maspin.
3. Altered activity and/or expression of components of the extrinsic pathway of apoptosis, which includes the tumor necrosis factor (TNF)- α family of ligands and the death receptors.
4. Altered activity and/or expression of transcription factors, such as NF- κ B, and p53, signaling pathways, such as the TGF- β , Akt, and stress-activated protein kinase (SAPK) cascade, and focal adhesion complex molecules, all of which ultimately modulate components of the intrinsic and extrinsic apoptotic pathways.

In this section, we will describe these mechanisms of apoptotic resistance and will define their significance in metastatic progression. The specific molecular pathways that contribute to these processes are schematically represented in Fig. 2.

4.1. Disruption of Caspase Function in Metastasis

Both the intrinsic and extrinsic apoptotic pathways lead to the activation of caspases, a family of cysteine proteases. Therefore, altering the activity of these genes

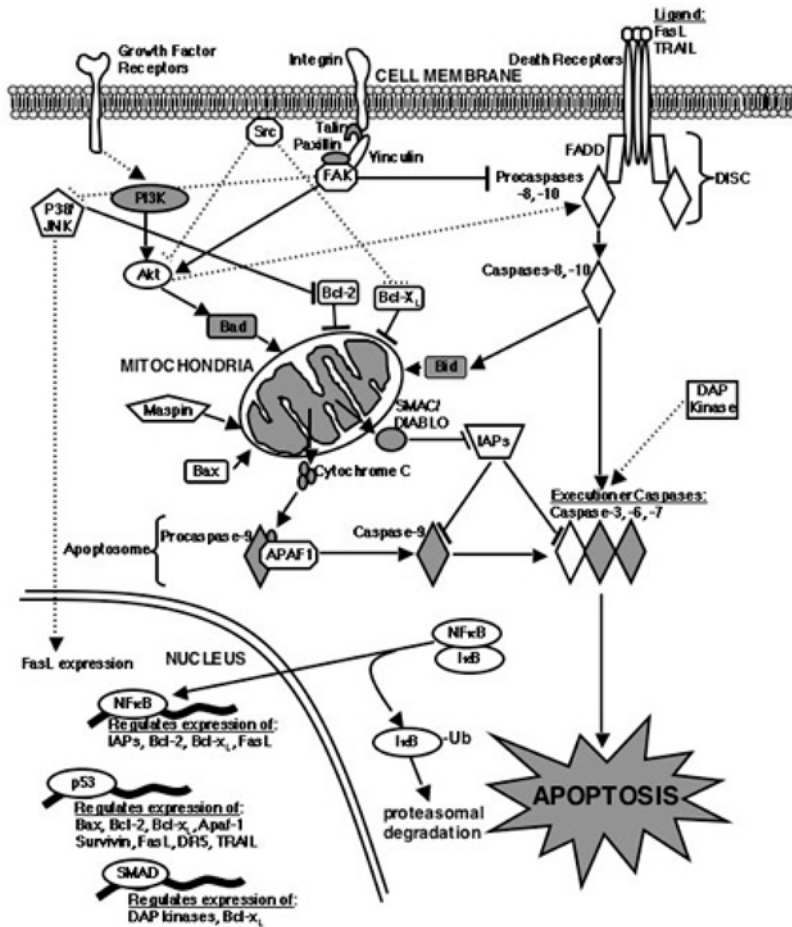


Fig. 2. Molecular signaling pathways regulating apoptosis. Signaling molecules are labeled and represented by shaded and unshaded shapes. An unshaded shape indicates that altered expression or activation of the protein is involved in metastatic spread, whereas a shaded shape indicates that there is no evidence for the protein's role in metastasis as of yet. Solid lines represent the positive (lines ending in arrowheads) or negative (lines ending in perpendicular line) regulatory interaction between proteins. Dashed lines represent a putative and/or indirect relationship between signaling molecules.

could significantly contribute to the process of apoptotic evasion. Caspase function may be disrupted in metastasis by mutation or diminished expression of caspase genes, overexpression of the IAPs, or reduced expression of DAP kinases.

4.1.1. Mutation and Altered Expression of Caspase Genes

The caspase family can be subdivided into two classes, the initiators and the effectors. The initiators include caspase-9, a key element of the protein complex central to the intrinsic/mitochondrial pathway of apoptosis called the apoptosome (Fig. 2), and the highly homologous caspases-8 and -10, components of the death-induced signal complex (DISC) complex downstream of the death receptors (Fig. 2) described later in this section. These initiators cleave and activate the primary effectors, caspases-3, -6, and -7.

As both apoptotic pathways converge on caspases, altered expression and/or activation of these proteases could impact signaling through both of these pathways. Caspases, however, are highly redundant, therefore disruption of a single caspase may be compensated for by other family members.

Mutation and altered expression of caspases have been associated with various forms of cancer, but most of these instances correlated with early events in tumor formation. Nonetheless, there are a few examples of caspase expression and mutation correlating with metastatic progression. In particular, apoptotic resistance of medulloblastoma cell lines may be attributed to loss of *caspase-8* and/or *caspase-10* mRNA expression resulting from aberrant promoter methylation (18,19). Analysis of medulloblastoma specimens also revealed that reduced *caspase-8* expression is a significant prognostic indicator of progression-free survival (20). *Caspase-10* mutations, which render the protein functionally defective, have been identified specifically in non-small-cell lung cancers (NSCLC) with lymph node metastases, when compared with NSCLC without metastases (21).

There is conflicting evidence for the role of caspase-3 in metastasis. Two retrospective studies comparing caspase-3 expression with clinical outcome of NSCLC indicated that caspase-3 protein levels, negatively correlated with lymph node metastasis (22,23). No correlation was found between caspase-3 transcript levels, apoptotic index, and tumor progression of hepatocellular carcinomas (HCC) (24), however; for gastric cancer, the data is conflicting. One group found that lower caspase-3 protein levels correlated with lymph node metastasis of gastric lymphoma (25). Another group found the opposite relationship—positive caspase-3 expression correlated with lymph node status—but they also showed that most of the caspase-3 protein in gastric carcinomas was not activated (26). Although caspases are clearly critical in manifesting apoptosis, most of the data linking reduced expression and mutation of caspases to apoptotic resistance and metastatic progression is indirect and correlative. Therefore, a causal relationship is speculative at best.

4.1.2. Altered Expression of Caspase Inhibitors of Apoptosis

IAPs are a family of caspase inhibitors that target both the intrinsic and extrinsic apoptotic pathways through specific inhibition of caspases-3, -7, and -9 (Fig. 2) (27). Eight members of the IAP family have been identified, including XIAP, cIAP1, cIAP2, and survivin. The best-characterized IAP, survivin, is overexpressed in malignancies of the lung, prostate, breast, colon, esophagus, pancreas, stomach, and central nervous system (CNS) (27–31). *Survivin* expression was significantly higher in prostate cancers with lymph node metastases (32); however, there was no significant relationship between survivin expression in colorectal cancer and risk for lymph node or distant metastases (30). In fact, decreased *survivin* expression was found in hepatic metastases in comparison with primary colon tumors (33). Thus, although a clear link between elevated *survivin* expression and transformation has been established, the contribution of survivin to metastasis is undefined.

4.1.3. Loss of Death-Associated Protein Kinase Expression is Related to Apoptotic Resistance

The DAP kinases are a family of calcium/calmodulin, serine/threonine kinases, which contain death-domains and are associated with the cytoskeleton (reviewed in refs. 34,35). The founding member of the family was identified in a functional genomics

screen, which involved selection for genes that confer resistance to interferon (IFN)- γ -stimulated apoptosis when expression was suppressed by antisense cDNA (36). DAP kinases promote apoptosis by acting downstream of the initiator caspase-8, but upstream of the effector caspases (Fig. 2) (37). Therefore DAP kinases, like IAPs, have the potential to affect both the extrinsic and intrinsic pathways of apoptosis. The best evidence for the role of DAP kinase in metastatic progression comes from Lewis lung carcinoma mouse model experiments (36). Loss of DAP kinase expression in murine Lewis lung cancer cells corresponded to enhanced metastatic behavior compared with syngeneic cells that express DAP kinase. When DAP kinase expression was restored in these highly aggressive lung cancer cells, their metastatic capacity was reduced in mice. The presumed cause of this reduced metastatic capacity was enhanced apoptosis of cancer cells as indicated by diminished *in situ* TdT-dUTP terminal nick-end labeling (TUNEL), a measure of DNA cleavage, of tumor sections (36).

Loss of DAP kinase expression also correlates with patient prognosis according to clinical data. IHC analysis of DAP kinase protein in 128 breast cancer specimens indicated that higher DAP kinase expression correlated with patient survival (38). Loss of DAP kinase expression may be linked to hypermethylation of its promoter (39). In NSCLC, methylation of the DAP kinase promoter was associated with an increase in patient tumor size and lymph node involvement (40). Furthermore, methylation-specific PCR was used to analyze ten brain metastases derived from different types of cancer. In nine of ten metastases, the DAP kinase promoter was hypermethylated, whereas normal brain tissue samples did not exhibit such hypermethylation (41). Although the DAP kinase family has only recently been identified, these studies suggest that it may be an important therapeutic target for conquering metastasis in the future.

4.2. Altered Signaling Through the Intrinsic Apoptotic Pathway in Metastasis

The intrinsic or mitochondrial apoptotic pathway relies on formation of a protein complex dubbed the apoptosome, composed of caspase-9, Apaf-1 and mitochondrial-derived cytochrome *c* (Fig. 2). Bcl-2 family members, as well as the serine protease inhibitor maspin, regulate cytochrome *c* release. Several components of this intrinsic pathway are differentially expressed in metastatic lesions.

4.2.1. Loss of Apaf-1 Expression in Metastasis

The reduced expression of Apaf-1 (apoptosis protease activating factor) has been linked to apoptotic resistance and metastatic spread of melanoma. Its down-regulation has been observed in metastatic melanoma cell lines compared with lines derived from primary melanoma tumors (42). Several metastatic melanoma cell lines exhibited *Apaf-1* promoter hypermethylation and epigenetic silencing of the gene (43). Loss of Apaf-1 protein in tumor tissue samples also appeared to correlate with metastases of melanoma (42). Recently, it has been demonstrated that *Apaf-1* haploinsufficiency, and therefore its diminished expression, increased with colorectal carcinoma progression and was associated with liver metastasis (44).

4.2.2. Altered Expression of the Bcl-2 Family

The Bcl-2 family consists of 25 pro- (Bax, Bak, Bid) and antiapoptotic (Bcl-2, Bcl-x_L) members that heterodimerize to form permeability pores in the mitochondrial membrane.

Proapoptotic family members promote cytochrome *c* release into the cytoplasm, prompting caspase activation and execution of the apoptotic program while antiapoptotic members prevent this cytochrome *c* release (Fig. 2) (45). Dysregulation of both pro- and antiapoptotic Bcl-2 family members occurs during cellular transformation, and upregulation of *Bcl-2* is linked to metastatic progression in vitro.

4.2.2.1. BCL-2

Examination of *Bcl-2* expression in a pancreatic tumor cell line and sublines of increasing metastatic potential revealed that progression from the low to highly metastatic state was accompanied by increasing levels of *Bcl-2* (46). Moreover, the metastatic variants developed resistance to apoptosis induced by a variety of biochemical stimuli. Furthermore, *Bcl-2* transfected parental cell lines produced a higher incidence of hepatic metastases in vivo as compared with the low *Bcl-2*-expressing parental cell line (46). Bcl-2 levels also correlated with prostate cancer progression. Relative to a slowly growing parental Dunning R-3327 rat prostate cancer cell line, rapidly proliferating, androgen-independent variants expressed higher levels of Bcl-2 protein (47). *Bcl-2* overexpressing murine melanoma cells experienced a 40% reduction in interleukin converting enzyme (ICE) and caspase protease activities and were more capable of surviving limited dilution cloning, indicating a decreased requirement for cell–cell contact (48). Related findings were reported in *Bcl-2*-transfected mitogen activated protein kinase kinase (MEK)-transformed mammary epithelial cells (EpH4) that were treated with the actin depolymerizing agent latrunculin A, which induces apoptosis (49). *Bcl-2* overexpression rescued parental cells from latrunculin-induced apoptosis, but was insufficient to enhance in vitro migration or invasion, hallmarks of metastasis. However, in mice inoculated with *Bcl-2* transfectants, the pulmonary metastatic burden was dramatically augmented, despite no differences in the kinetics of primary tumor development (49). These studies suggest that there are distinct mechanisms driving primary tumor formation and metastasis and that Bcl-2-mediated inhibition of apoptosis is one such pathway.

It is hypothesized that Bcl-2 dysregulation and inhibition of apoptosis may expose breast tumor cells to further genetic damage, leading to accumulation of genetic alterations that increase the risk of metastases (50). In an analysis of T₁ primary breast tumors, intense Bcl-2 staining predicted ninefold excess odds for lymph node metastasis when compared with Bcl-2 negative tumors (50). Bcl-2 expression was inversely correlated to histological grade and Bcl-2 positive tumors had a lower apoptotic index, supporting a relationship between high Bcl-2 expression, resistance to apoptosis, and concomitant metastasis. In direct contrast, Le et al. (51) reported that Bcl-2 expression was associated with a low risk of distant metastases of breast cancer. Confounding issues were discrepancies in staining methods, duration of follow-up, and consideration of overall relapse versus specific types of relapse (51). Thus, the role of Bcl-2 expression in breast cancer metastasis has yet to be clearly defined.

High Bcl-2 levels are associated with prostate cancer progression. By tissue microarray analysis, significant Bcl-2 overexpression was found in hormone refractory prostate cancer and prostate cancer metastases in comparison with localized prostate cancer (52). Likewise, lymph node and bone metastases from prostate cancer exhibited statistically significant increases in Bcl-2 staining compared with prostatic intraepithelial neoplasia and localized prostate cancer (47).

4.2.2.2. BCL-x_L

Another member of the Bcl-2 family with an emerging role in metastatic progression is Bcl-x_L. *Bcl-x_L* overexpression and apoptotic resistance are often linked to *Bcl-2* expression levels, but it is evident that these proteins have nonredundant functions in apoptosis and metastasis. For example, only *Bcl-x_L* has been linked to amorphosis (programmed cell death that occurs upon disruption of the actin cytoskeleton) resistance. In a cytoskeletal-based functional screen to identify genes bestowing resistance to amorphosis, the *Bcl-x_L* coding sequence alone conferred amorphosis resistance to sensitive cells (53).

Numerous in vitro and in vivo transfection studies confirm the significance of *Bcl-x_L* in tumor metastasis and highlight Bcl-x_L's role in several distinct aspects of metastasis. *Bcl-x_L*-overexpressing breast carcinoma cells were less adherent to laminin, fibronectin, and collagen IV than vector-transfected controls (16,54). Cell viability in suspension, a measurement of anoikis sensitivity, was drastically enhanced in *Bcl-x_L* overexpressing MDA-MB-435 cells (54). Proficient survival in suspension also translates to improved viability in the circulation. In a competition assay in which a mix of control and *Bcl-x_L*-transfected MDA-MB-435 cells were injected into the tail vein of nude mice, there was marked enrichment of viable *Bcl-x_L* overexpressing cells in the vasculature over a period of 4 h (54). MDA-MB-435 cells overexpressing *Bcl-x_L* were also several-fold more resistant to transforming growth factor (TGF)-β- and TNF-α induced apoptosis than control transfectants, and this phenotype was abrogated by *Bcl-x_L* antisense (55).

Intramammary fat pad implantation of *Bcl-x_L* transfected MDA-MB-435 cells resulted in a discernable rise in the incidence and size of lung metastases (16). Furthermore, lymph node metastases were present in mice implanted with *Bcl-x_L* overexpressing MDA-MB-435 cells, but not in control mice (54). Quite striking is the demonstration that breast cancer cells overexpressing Bcl-x_L exhibited heightened genomic instability as measured by genomic fingerprinting. This genetic instability sequentially increased from the *Bcl-x_L*-overexpressing primary tumor to the metastases found in bone, lymph nodes, and lungs of nude mice (56). Moreover, *Bcl-x_L* overexpression in primary breast cancer has been associated with high tumor grade and increased risk for nodal metastases (57). Together, these studies suggest that dysregulation of the antiapoptotic molecule Bcl-x_L has consequences on the genomic level that can affect the apoptotic and cellular processes contributing to metastatic tumor cell survival.

4.2.2.3. BAX

Bax is a proapoptotic Bcl-2 family member that translocates to the mitochondria and promotes the release of cytochrome *c* and Smac/Diablo, a negative regulator of IAPs (Fig. 2). It has been proposed that high expression of *Bax* results in a greater propensity for tumor cells to undergo apoptosis, thereby curbing metastasis and providing a better prognosis for the patient.

Acquisition of a prosurvival phenotype characterized by low-*Bax* expression accompanies tumor cells in their progression to metastasis. Metastatic variants derived from lymph node metastases of prostate cancer were more resistant than parental or non-metastatic cells to pharmacologically induced apoptosis, and displayed delayed kinetics of the classic hallmarks of apoptosis, DNA fragmentation, and PARP cleavage (58). Notably, the highly metastatic variants expressed low-steady-state *Bax* levels when compared with the parental and non-metastatic variants.

Given the above in vitro data, it is not surprising then that several studies predicted Bax to be a useful clinical prognostic indicator. IHC performed in 119 primary tumors from women with metastatic breast adenocarcinoma revealed heterogeneous Bax negativity compared with the almost uniform Bax positivity found in non-neoplastic epithelium and carcinoma *in situ* (59). Patients with Bax negative tumors had significantly shorter time to progression (6.6 mo compared with 10.6 mo in Bax positive patients) and reduced overall survival (8.1 mo vs 15.7 mo). In patients with colorectal carcinoma, Bax expression was significantly correlated with less lymph vessel invasion, less depth of invasion, and better prognosis (5-yr survival rate) (60). IHC of primary tumors from patients with oral and oropharyngeal squamous cell carcinoma indicated that Bax positivity was greater in the lower disease stages T₁/T₂ than in the more advanced T₃/T₄ stages (61). Even more convincing, Bax positivity was significantly elevated in patients without lymph node metastases than in patients with lymph node metastases, and these Bax-positive patients experienced improved overall 5-yr survival (61).

Expression of other Bcl-2 family members in conjunction with Bax can also be valuable potential prognostic markers. Together, low Bax and high Bcl-2 staining were associated with low apoptotic index, high tumor grade, axillary lymph node involvement, and metastasis in patients with primary breast carcinoma (62). Whether these analyses will result in the development of Bax-directed therapies for metastatic cancer remains to be seen.

4.2.3. Maspin

The serpin (serine protease inhibitor) maspin has garnered increasing interest as a molecular marker and potential therapeutic target in several types of cancer, but most especially in breast cancer (reviewed in ref. 63). Mounting evidence suggests that maspin sensitizes cells to apoptosis via the mitochondrial pathway. Overexpression of *maspin* increased Bax and activated Caspases-8 and -9 in breast and prostate cell lines (64,65). Conversely, Bax and Caspase-8 and -9 repression eliminated sensitization to apoptosis-inducing drugs via maspin in these cell lines (65). Additionally, Bcl-2 overexpression protected endothelial cells from maspin-mediated apoptosis in vitro (66). A recent report suggested that maspin regulation of mitochondrial apoptosis might be direct. Latha et al. showed that maspin translocates to the mitochondrial membrane, similar to Bcl-2 family members, altering the mitochondrial membrane potential and leading directly to cytochrome *c* release (67). Thus far, maspin is the only serpin shown to induce apoptosis and actually, its extracellular serine protease inhibitor activity is not involved in its apoptosis-promoting activity (64).

Several mouse models implicate maspin-mediated apoptosis in metastatic progression (68). Transgenic mice expressing *maspin* in mammary epithelial cells were crossed to an oncogenic SV40 T mouse strain, which always develop mammary tumors. Progeny showed reduced primary tumor growth and incidence of pulmonary metastases. The observed reduction can be attributed to enhanced apoptosis of cancerous mammary epithelial cells, at least in part (decreased angiogenesis and motility of malignant cells may also contribute) (69). A xenograft model using the highly metastatic human breast cancer cell line TM40D transfected with *maspin*, again showed that *maspin*-expressing tumor cells had reduced primary tumor growth and were incapable of supporting pulmonary metastasis formation (70). Finally, polyoma middle T oncogenic mice were allowed to develop primary mammary tumors and were then administered maspin protein via liposome carriers. Primary tumor growth and lung metastases were inhibited in maspin-treated mice (71).

In clinical correlates, maspin expression has also been linked to cancer aggressiveness. Downregulation of maspin has been reported in invasive and metastatic breast carcinoma samples. IHC analysis of a large cohort of breast cancer tissue specimens revealed a stepwise diminution of maspin expression as tumors progress from *in situ* to invasive to lymph node metastasis-positive (72,73). Maspin expression was also negatively associated with invasive depth and metastasis in gastric carcinomas (74).

Several lines of evidence suggest that the diminished *maspin* expression observed in metastatic lesions may be attributed to hypermethylation of its promoter. Breast and gastric cancer cell lines treated with the demethylating agent 5-aza-2'-deoxycytidine (5-aza C) had enhanced *maspin* expression (75–77). Novel microarray and chromatin immunoprecipitation techniques provided direct evidence for *maspin* promoter methylation in pancreatic cancer (78,79). There is a growing body of evidence for the role of maspin in the intrinsic pathway of apoptosis and in metastasis suppression, suggesting that targeted re-expression with of this serpin with demethylating agents may be a logical approach for the treatment of apoptotic resistance of metastases.

4.3. Alteration of the Extrinsic Apoptotic Pathway in Metastasis

The extrinsic pathway of apoptosis is mediated by death receptors of the TNF- α receptor family. This pathway is activated by ligand binding to one of the death receptors, followed by formation of the DISC, which is composed of Fas-associated death domain containing protein (FADD), and caspase-8 or -10. DISC then either signals downstream effector caspases (3, 6, and 7) leading directly to cell demise or signals through the Bcl-2 family member Bid and the intrinsic, mitochondrial pathway of cellular apoptosis (Fig. 2). Two ligands of the TNF- α family have been implicated in progression of cancer cells toward a metastatic phenotype—FasL and TNF-related apoptosis-inducing ligand (TRAIL).

Resistance to FasL and TRAIL-mediated apoptosis can be conferred by multiple mechanisms. The mechanisms specific to these ligands will be explained below, but disruption of the DISC complex can potentiate both FasL and TRAIL resistance. Somatic mutations in *FADD* that functionally disrupt the protein have been observed at a higher frequency in metastatic NSCLC compared with primary tumors (21). *Caspase-8* and *-10* mutations have been implicated in TRAIL resistance in medulloblastoma and NSCLC (18–21). Some general apoptotic and survival pathways that have previously been discussed or will be discussed later in this chapter can mediate FasL and TRAIL resistance, as well. The transcription factor p53, which is mutated in 50% of cancers, regulates expression of both *TRAIL* and *FasL*, as well as the Fas receptor (80). Several lines of evidence also suggest that mutation of the Bcl-2 family member *Bax*, which occurs frequently in mismatched-repair deficient tumors, also contributes to TRAIL-induced apoptotic resistance (81,82). Other signaling pathways such as TGF- β , protein kinase C (PKC), Akt cell survival, and mitogen-activated protein kinase (MAPK) pathways affect sensitivity of cancer cells to TRAIL and FasL induced apoptosis (83–86).

4.3.1. Fas/FasL-Specific Mechanisms of Apoptotic Resistance

The death receptor ligand FasL is normally expressed by cytotoxic T lymphocytes and NK cells and is involved in immunosurveillance of tumor cells that are expressing the Fas receptor. Fas/FasL interactions have been shown to be involved in suppression of melanoma metastasis (87). *FasL*-deficient mice had a higher incidence and number of

spontaneous metastases compared with wild-type syngeneic mice injected with K1735 murine melanoma cells.

Whereas Fas/FasL signaling is important in suppressing metastasis, metastatic tumor cells are frequently resistant to Fas-induced apoptosis. Hedlund et al. studied seven prostate cancer cell lines for sensitivity to Fas-mediated apoptosis. Cell lines derived from primary tumors were more sensitive to apoptosis and the four most resistant lines were derived from metastatic lesions (88). Similarly, Fas resistance has been observed in pancreatic ductal carcinoma cell lines and osteosarcoma cell lines with differing metastatic potentials (89,90).

Multiple mechanisms have been implicated in FasL-resistance of the metastatic cell, including reduced expression of the Fas receptor. IHC, and RT-PCR analysis of colorectal cancer specimens revealed that Fas expression was reduced in colorectal adenomas, colorectal carcinomas, and liver metastases compared with matched primary colorectal carcinomas (91). Similarly, Wang et al. found that Fas levels were significantly lower in metastatic liver lesions compared with matched primary colorectal tumor specimens (92). In HCCs, Fas levels correlated with disease-free survival. Fas-positive HCCs exhibited reduced incidence of intrahepatic metastatic foci and enhanced incidence of apoptotic tumor cells (93). Furthermore, poor patient prognosis has been correlated with Fas:FasL ratios in hepatic and ovarian carcinomas (94,95).

Another mechanism of Fas-mediated apoptosis resistance is enhanced expression of soluble Fas (sFas). The sFas receptor is released extracellularly and will compete for binding to FasL expressed by cytotoxic T cells and NK cells, thus blocking ligand engagement of Fas-expressing tumor cells. One study indicated that it is not tumor cells that have altered their sFas levels, but it is the surrounding noncancerous hepatocytes and mononuclear liver cells that express sFas to permit metastatic growth in the liver (93). Circulating sFas concentration in breast cancer patients' serum was measured by enzyme-linked immunosorbant assay (ELISA). High-sFas patients led to poorer prognosis for both overall and disease-free survival (96). Similarly, another study found sFas plasma levels to be a significant independent predictor of poor overall survival of metastatic breast cancer (97). In addition, serum levels of sFas were higher in patients with advanced melanoma that were refractory to chemotherapy (98). Expression of the FasL decoy receptor DRc3 similarly affects FasL-initiated apoptosis by antagonizing ligand binding to Fas. Wu et al. determined that DcR3 positively correlated with malignancy in tumor patients and is associated with tumor differentiation, lymph node, and metastatic status (99).

In perhaps the most aggressive mechanism of tumor self-defense, metastatic cells have acquired a way to use the host's Fas/FasL immune defense against it in what has been termed the "tumor counterattack phenomenon." Tumor cells can express FasL on both their cell surfaces and in a soluble, extracellular form, which can then induce programmed cell death in neighboring tumor-infiltrating lymphocytes (TILs). High FasL expression levels are associated with lymph node metastasis grade in breast carcinoma (100). Mann et al. provided evidence for FasL involvement in colorectal carcinoma evasion of TILs (101). They determined FasL expression and CD25 levels (a measure of lymphocyte activation) in matched primary tumor and liver metastases from 21 colorectal carcinoma patients. FasL was detected in 30 to 40% of tumors, but in 60 to 100% of metastases. The level of lymphocyte activation was lower in 90% of the metastases (101). As further evidence of the FasL-mediated mechanism of immune evasion in colorectal

cancer, Okada et al. found that the *in situ* nick translation labeling index (a measure of apoptosis) of TILs from 41 colorectal patients was higher in colorectal carcinomas with lymph node metastases versus those without metastases (102). In addition, increased apoptosis of Fas-positive TILs was observed in metastatic gastric carcinoma (103).

4.3.2. TRAIL Specific Mechanisms of Apoptotic Resistance

TRAIL is highly homologous to FasL, but unlike FasL, it is more widely expressed in a variety of tissues. TRAIL has five known receptors, two of which are complete with death domains (DR4 and DR5), whereas the other three are decoy receptors missing functional death domains (DcR1, DcR2 and OPG). TRAIL has garnered much interest as a potential therapeutic cancer target as tumor cells are more susceptible to TRAIL-induced cell death than normal epithelial cells, presumably because tumor cells express DR4 and DR5 at higher levels. Normal cells may express higher levels of the decoy receptors, thus protecting them from TRAIL-mediated apoptosis (104).

Although its function is not completely understood, TRAIL expression on liver NK cells appears to play a role in tumor cell surveillance. Mice depleted of NK cells or treated with a TRAIL-neutralizing antibody showed a significant increase in spontaneous liver metastases (105). Cretney et al. developed a mouse model of *TRAIL* deficiency (106). *TRAIL* gene-targeted mice have an absence of *TRAIL* expression in liver and spleen mononuclear cells and an absence of TRAIL cytotoxicity. These mice were more prone to spontaneous metastases, showed decreased response to the antimetastatic effects of therapeutic doses of interleukin-12, and α -galactosylceramide, and were more sensitive to the carcinogen methylcholanthrene (106). In ovarian cancer tissue specimens, *TRAIL* expression correlated with patient survival (107). As we shall see in the latter part of this chapter, *TRAIL* replacement by gene therapy in mice can effectively suppress tumor growth and experimental metastases (108–110).

TRAIL is clearly an important and an effective molecule for eliminating cancerous, even metastatic cells; however, TRAIL resistance has been detected. Several melanoma cell lines exhibit resistance to TRAIL and interestingly, primary oral cancer cell lines are more susceptible to TRAIL-induced apoptosis than their metastatic counterparts (111,112). There are several mechanisms of apoptotic resistance that are unique to the TRAIL pathway. Mutation of TRAIL receptors *DR4* and *DR5* and a higher incidence of allelic loss of the TRAIL receptors have been detected in breast cancers with metastases. Transfection of the mutated receptors found in these breast tumors suppressed TRAIL-induced apoptosis (113). Loss of receptor expression has also been associated with TRAIL resistance in some melanoma cell lines (111).

Tumor cell expression of decoy receptors is an alternate mechanism of resistance to TRAIL apoptosis. Zhang et al. found that TRAIL-resistant melanoma cell lines express high levels of DcR1 and DcR2, which may antagonize TRAIL binding to the complete TRAIL receptors (111). DcR2-positive metastatic gastric carcinoma cells were also identified in patient ascites. These cells were resistant to TRAIL-induced apoptosis (114). Osteoprotegerin (OPG), the other decoy receptor, is expressed in bone stroma cells of prostate cancer patients with detectable bone metastases. A prostate cancer cell line treated with media conditioned by the patient derived bone stroma exhibited reduced sensitivity to TRAIL-induced apoptosis, suggesting that expression of OPG by bone stroma may create a permissive environment for bone metastasis colonization (115).

4.4. Transcriptional Regulators of Apoptotic Resistance in Metastasis

Whereas apoptotic mediators contribute to tumor aggressiveness and metastasis by virtue of their direct effects on the intrinsic and extrinsic death pathways, transcription factors are indirect but pivotal metastatic regulators through their pleiotropic effects on gene regulation (Fig. 2). Modulation of genes involved in apoptosis, together with genes associated with invasion, angiogenesis, proliferation, and survival, identify transcription factors as attractive targets for therapeutic intervention. Several such factors are outlined below.

4.4.1. NF- κ B

In its inactive state, NF- κ B is bound in the cytoplasm by I κ B. I κ B phosphorylation leads to its ubiquitination and proteasomal degradation, thereby releasing free, activated NF- κ B. Activated NF- κ B then translocates to the nucleus where it promotes transcription of a myriad of antiapoptotic genes including *Bcl-2*, *Bcl-x_L*, *Traf-1*, *Traf-2*, *survivin*, *cIAP-1*, *cIAP-2*, *c-FLIP*, *FasL*, and *p21*, as well as genes implicated in angiogenesis and invasion such as *VEGF*, *iNOS*, *MMP2*, *MMP9*, and *uPA* (116,117) (Fig. 2).

As cells progress to a metastatic state, there is constitutive activation of NF- κ B and target genes, resulting in an apoptotic resistant phenotype. Microarray analysis of primary murine keratinocytes, transformed PAM212 cells, and lymph- or lung-metastatic sublines indicated that approximately half of the genes differentially enriched in the metastatic variants represented NF- κ B-related genes—either genes implicated in NF- κ B regulation (ubiquitin activating enzyme E1 which degrades I κ B), or genes that are NF- κ B targets (*cIAP1*, *FasL*) (118). Similarly, enhanced activation of the NF- κ B pathway was found in mammary cells undergoing an in vitro correlate of breast cancer progression, the epithelial–mesenchymal transition (EMT). In a comparison between transformed mammary epithelial cells (EpRas) and those that undergo EMT in response to TGF- β (EpRasXT), enriched expression of NF- κ B target genes was demonstrated in EpRasXT cells (119).

In human breast tumors, there is a substantial and striking correlation between constitutive NF- κ B activation and progression to a more aggressive state, characterized by hormone-independent growth. NF- κ B was constitutively activated in 50% of primary breast tumors (120). Furthermore, those tumors with enhanced NF- κ B binding activity were estrogen receptor (ER) negative. In an independent analysis, high NF- κ B activity was observed in ER[−], ErbB2⁺ human breast tumors. Blockade of NF- κ B signaling, and addition of heregulin to the ER[−], ErbB2⁺ SKBR3 cell line dramatically increased apoptosis (121). The connection between constitutive NF- κ B activity and the poorly differentiated, highly metastatic ER[−], ErbB2⁺ phenotype emphasizes the significance of NF- κ B signaling in the pathway to aggressive disease. These studies also highlight NF- κ B as a prospective molecular candidate for inhibition.

Suppression of NF- κ B activity can inhibit metastasis without affecting tumorigenicity in vivo. In an orthotopic nude mouse model, all of the mice injected with either the murine pancreatic cancer cell line AsPc-1 or stable transfectants of this cell line expressing a hyperstable I κ B mutant developed pancreatic tumors (122). In the parental AsPc-1-tumor bearing mice, 8/11 mice developed liver metastases and ascites, and all mice had peritoneal metastases. Remarkably, only one-tenth of AsPc-1/I κ B-mutant bearing mice developed peritoneal metastases and ascites, and none of these mice developed hepatic metastases.

Pharmacological inhibition of NF- κ B activity can be achieved through proteasome inhibitors that block I κ B degradation and other compounds that inhibit I κ B

phosphorylation, such as BAY 11-7082 and BAY 11-7085. The specific 20S proteasome inhibitor PS-341 impeded NF- κ B activation, slowed the proliferation of human small cell lung cancer cell lines in vitro and in BALB/c mice, and limited angiogenesis (123). NF- κ B inhibition renders cells susceptible to apoptosis during tumor cell readherence and implantation. The human colorectal cancer cell lines HT-29 and DLD-1 were transiently suspended and allowed to readhere in vitro while in the presence of BAY 11-7085 (124). Augmentation of apoptosis occurred in the presence of drug, and an even more impressive reduction of the apoptotic threshold was found when cells were transduced with an I κ B super repressor construct specifically targeting NF- κ B. Moreover, BAY 11-7085 reduced colon cancer cell implantation in an in vivo model of metastasis (124), highlighting the potential therapeutic utility of NF- κ B inhibition.

4.4.2. p53

Another transcription factor fundamentally important to apoptosis and metastasis is the tumor suppressor p53, which regulates hundreds of genes through transactivation or repression. Activation of p53 can trigger a plethora of biological outcomes including cell-cycle arrest, apoptosis or anoikis, senescence, and differentiation. These outcomes are influenced by cell context, target gene selectivity, covalent posttranslational modifications of p53 itself, and activation of the proteins with which p53 interacts (125). Proapoptotic genes positively regulated by p53 include members of the death receptor families *Fas* and *DR5* as well as mitochondrial associated proteins *Bax*, *Bak*, *Noxa*, *Puma*, *Pig3*, and *Apaf-1* (126,127). p53 also represses the promoters of *Bcl-2*, *Bcl_{x_L}*, and *survivin*, all antiapoptotic molecules (Fig. 2).

Although the contribution of p53 to apoptosis and cell-cycle control is apparent, the mechanisms whereby p53 affects metastatic growth are less understood. In *ras*⁺ *myc*⁺ initiated mouse prostate tumors, loss of p53 function lead to widespread metastases (128). In mouse embryo fibroblasts transformed with E1A and *ras* (C8), p53 wild-type cells underwent apoptosis upon detachment from the substratum; however, those cells with dominant negative or null p53 were conferred a survival advantage (129). In an elegant in vivo experiment, athymic nude mice were injected with C8 fibroblasts containing either wild-type or mutant p53, lungs were either isolated within 2 to 4 hs or after several wk. Cells isolated from the lungs 2 to 4 h following injection were predominantly p53 mutant, and accordingly, the majority of lung macrometastases carried mutant *p53* alleles. Thus, loss of p53-mediated apoptosis and improved tumor cell survival in the vasculature provide a foundation for metastatic growth. Loss of the proapoptotic function of p53 also renders cells more genetically unstable. There is a substantial correlation between human tumors bearing null or mutant p53, lymph node metastasis, and mutations in *Her2*, *EGFR*, and *myc* (130). Hence, stimulation or restoration of wild-type p53 function has potential therapeutic implications, and these will be addressed later in this chapter.

4.4.3. TGF- β Signaling

TGF- β signaling affects numerous biological processes such as cell-cycle progression, developmental regulation, chemotaxis, adhesion, angiogenesis, growth suppression, and apoptosis (131). Although clearly a master regulator of metastasis, the definitive role of TGF- β in tumor cell progression and metastasis is quite complex. In the early stages of carcinogenesis, TGF- β is thought to function as a tumor suppressor,

especially in normal, nontransformed tissues. However, during progression to a more invasive metastatic state, TGF- β signaling sensitivity is attenuated and accompanied by enhanced TGF- β stromal secretion, resulting in a switch from tumor suppressor to metastasis promoter. The mechanisms underlying when and how this complex transition occurs are uncertain, but may involve gene dosage effects in the TGF- β signaling pathway and aberrant activation of the Ras/MAPK or PI3K pathways (132).

TGF- β binding induces complex formation between the TGF- β type II receptor (T β RII) and the type I receptor (T β RI). T β RII transphosphorylates T β RI, which subsequently phosphorylates transcriptional activator SMAD proteins. SMADs form heterodimeric complexes and translocate to the nucleus where they bind DNA directly or associate with other transcription factors and modulate expression of genes such as *DAPK*, *c-myc*, *c-Jun*, *p21*, and *VEGF* (133). What largely determines the TGF- β -mediated transcriptional response are environmental cues, cell context, and developmental stage (133). TGF- β signaling is implicated in initiation, integration, and execution of apoptosis by cooperating with Fas and TNF- α , activating the SAPK/JNK cascade, and down-regulating Bcl_{x_L}/Bcl-2 while activating caspases- 1, 3, 8, and 9 (131).

Gene expression profiling of normal human renal epithelium, non-metastatic renal cell carcinoma (RCC), and primary and disseminated lesions showed a reduction in both T β RII and TGF- β -regulated genes in RCC and to a greater degree in metastatic lesions (134). T β RII expression was reduced in 1 out of 16 primary RCC tumors that did not produce metastases, but was reduced in 8 out of 9 primary tumors in which metastases were present (134). Similarly, microarray analysis of a NSCLC cell line and two highly metastatic sublines revealed reduced *SMAD4* expression along with alterations in genes involved in invasion and apoptosis in the metastatic sublines (135). These data suggest that escape from apoptosis and attenuation of TGF- β -mediated signaling are potential mechanisms influencing metastatic progression in cancers such as RCC and lung carcinoma.

Both in vitro and in vivo evidence support TGF- β signaling desensitization as a driving metastatic force. This can occur through loss of T β RII function itself, or through loss of downstream signaling. Transgenic mice bearing the dominant negative T β RII mutant that disrupts TGF- β signaling developed larger and more frequent lung and liver metastases than parental mice despite no change in primary tumor size (136). Defects in TGF- β signaling also contribute to the metastatic phenotype by altering anoikis sensitivity. Upon restoration of functional TGF- β signaling in the human breast carcinoma cell line MDA-MB-468, cells lost several phenotypic markers characteristic of invasion and metastasis. They underwent rapid apoptosis marked by caspase activation, became sensitized to anoikis, and lost the ability to survive in soft agar (137). Disturbance of the apoptotic machinery via disruption of TGF- β signaling fosters an environment apt for matrix-independent survival, invasion, and subsequent metastasis.

There are translational implications for loss of TGF- β signaling and metastatic progression. A somatic missense mutation in the catalytic core of T β RI that inactivates TGF- β signaling was identified in lymph node metastases from primary breast cancer (138). In prostate cancer, poor clinical outcome was associated with overproduction of TGF- β and loss of T β RII (139). However, increased expression of TGF- β was widespread in colorectal and gastric carcinoma, highlighting the dual functions of TGF- β signaling and its resultant complexities in human cancer (140). Many other examples exist in which genetic or epigenetic inactivation of *T β RI*, *T β RII*, or the *SMADs* was found in

human tumors (140), exemplifying the concept that inactivation of the TGF- β signaling pathway is a fundamental route to conferring survival advantages upon tumor cells.

4.5. Resistance to Anoikis

Epithelial cells normally adhere to a basement membrane. Loss of this cell adherence results in a type of programmed cell death referred to as anoikis. As described above, surviving this loss of cell–cell adherence is critical for the spread of metastasis, as tumor cells must disengage themselves from the primary site of tumor growth to enter circulation. Therefore resistance to anoikis is key to metastatic progression. Many highly metastatic cell lines, including melanoma, gastric carcinoma, and oral squamous cell carcinomas exhibit anoikis-resistance in vitro (141–143).

Known mechanisms of anoikis resistance involve activation of the PI3K-Akt pathway, a cell survival pathway that culminates by upregulating many of the proteins involved in cell death previously described, including Bcl-2 family members and components of the extrinsic apoptotic pathway (Fig. 2). Anoikis resistance of Ras-transformed cells can be completely explained by activation of the PI3K/Akt pathway (144). Additionally, in human colorectal cancer tumor specimens, Akt activating-phosphorylation has been found to be negatively associated with apoptosis and positively correlated with stages of malignant progression, such as invasive depth and lymph node status (145).

Anoikis may also be manifested by coordinated downregulation of the SAPK pathway, incorporating either MAP kinases p38 or JNK (Fig. 2). As evidence of a functional link to anoikis resistance, SAPK inhibitors have been shown to abolish anoikis and enhance cell adherence (146). These SAPKs also converge on many of the apoptotic regulators that we have already addressed. Evidence suggests that JNK phosphorylation inhibits Bcl-2 function thus promoting apoptosis (147). Additionally, the p38 pathway has been shown to upregulate *FasL* expression (148). In this section, we will briefly describe the upstream components that regulate the Akt and SAPK pathways to avoid anoikis.

Integrins are cell surface proteins that interact with components of the extracellular matrix and upregulate Akt and downregulate SAP kinases. The $\alpha 4$ integrin is expressed in a number of metastatic melanomas and osteosarcomas. Engagement of $\alpha 4$ integrin with a monoclonal antibody (MAb) increased anoikis of a human osteosarcoma cell line in vitro (149). In another example of integrin signaling involvement in metastatic progression, periostin, which binds $\alpha 5\beta 3$ integrins thus enhancing Akt activation and cell survival, was overexpressed in 80% of colorectal cancers, with the metastatic tumors exhibiting the highest expression (150).

Integrins interact with the actin cytoskeleton through a multiprotein complex called the focal adhesion complex consisting of talin, vinculin and paxillin (Fig. 2). Cancer cells lacking *vinculin* have been shown to be highly metastatic (151,152) and murine embryonic carcinoma cells devoid of vinculin are resistant to anoikis. The observed increase in survival in vinculin null cells may be attributed to enhanced recruitment of focal adhesion kinase (FAK) to the focal adhesion complex (153). The nonreceptor tyrosine kinase FAK is an important regulator of anoikis. Its expression positively correlates with metastasis in a wide variety of cancers. In a mouse xenograft model, *FAK* expression was silenced by RNA interference in pancreatic adenocarcinoma cells. *FAK* gene silencing promoted anoikis and resulted in decreased metastasis in mice (154). Anoikis resistance in FAK-activated malignancies may be mediated by downregulation of p38 signaling

(146). However, FAK may also potentially promote anoikis resistance by sequestering a component of DISC, thus blocking the extrinsic apoptotic pathway (155). A definitive mechanism of FAK-mediated anoikis resistance has yet to be resolved.

Anoikis resistance can also be attributed to hyperactivation of another nonreceptor tyrosine kinase, Src. In human colon cancer cell lines, Src expression and activity corresponded with increased Akt phosphorylation and anoikis resistance (156). Ectopic expression of kinase-defective mutants of Src in human metastatic colon cancer cells resulted in enhanced sensitivity to apoptosis and appeared to be associated with a decrease in *Bcl-x_L* (157). The Src substrate p130 (Cas), which is normally dephosphorylated and inactivated upon cell detachment in anoikis-sensitive cells, is not dephosphorylated in anoikis-resistant lung adenocarcinoma cells. This constitutive p130 (Cas) phosphorylation is dependent on Src activity (158).

Growth factor receptors can also mediate anoikis resistance by a variety of molecular mechanisms. Anoikis-sensitive head and neck squamous cell carcinoma (HNSCC) could be protected from cell death upon loss of cell adherence by the hepatocyte growth factor. The hepatocyte growth factor receptor (c-met) has a well-established role in metastasis progression. The observed anoikis resistance is dependent on Akt and on the MAPK Erk, which regulates cell proliferation (159). EphrinA2 is another receptor tyrosine kinase that can suppress anoikis. Overexpression of *Ephrin A2* in pancreatic adenocarcinoma cell lines resulted in anoikis resistance and enhanced cellular invasiveness in vitro, whereas *EphrinA2* silencing allowed for anoikis in vitro and reduced tumor growth and metastasis in vivo (160). EphrinA2 anoikis resistance may be FAK-mediated as FAK phosphorylation corresponds to EphrinA2 activity. The neurotrophin receptors, or Trk receptors, also promote anoikis resistance. TrkB was identified in a functional screen for genes that suppress anoikis when they are overexpressed (161). *TrkB* ectopically expressed in nonmalignant epithelial cells led to anoikis-resistance in vitro and rapidly growing and metastasizing tumors when injected in mice. This resistance is mediated by constitutive activation of the PI3K-Akt survival signaling pathway (161) and *TrkB* is frequently overexpressed in a variety of highly aggressive human malignancies (162–164). As further evidence of the connection between Trk receptors and metastasis, a Pan-trk inhibitor CEP-701 inhibited metastatic colonization and enhanced overall survival in a rat prostatic cancer model (165).

We have not exhaustively cataloged the molecular mechanisms of anoikis resistance, but we have identified many of the key players. Other growth factors and cell-adhesion molecules that affect Akt, p38 and/or FAK or Src activity might also contribute to anoikis resistance.

5. Resistance to Pharmacologically Induced Apoptosis

As described earlier in this chapter, the ability of cancer cells to evade apoptosis during dissemination and colonization contributes to the development of metastases. In a similar vein, the ability of these same cells to resist pharmacologically induced apoptosis is a conduit for metastatic propagation. Often the cells that are highly resistant to chemotherapy are those with the highest metastatic propensity. Outlined below are three agents well characterized for their role in apoptosis resistance and metastasis. This discussion is by no means exhaustive, but rather, highlights specific instances in which highly metastatic cells are also those that are highly resistant to exogenously or pharmacologically

induced apoptosis. A greater understanding of how metastatic cells develop resistance to pharmacologically induced apoptosis may improve our strategies for the prevention or treatment of metastasis.

5.1. 5-Fluorouracil and Derivatives

The fluoropyrimidine 5-fluorouracil (5-FU) is the most widely used chemotherapeutic agent for the treatment of colorectal cancer ([166](#)). 5-Fluorouracil and its derivatives UFT (composed of a 5-fluorouracil analog, tegafur, and uracil) and S-1 are cytotoxic agents that inhibit thymidylate synthase, a key enzyme in the production of thymidylate. DNA synthesis in the absence of thymidylate leads to a futile cycle of deoxyuridine incorporation into and excision from DNA, leading to an apoptotic “thymineless” death ([167](#)).

Several mechanisms can be attributed to fluoropyrimidine resistance, among which are increased thymidylate synthase expression and intrinsic pharmacokinetic resistance. Significantly higher levels of thymidylate synthase mRNA were found in hepatic metastases from patients resistant to fluoropyrimidine-based therapy ([168](#)). Another potential mechanism responsible for 5-FU resistance is apoptotic resistance. Although the biochemistry of the fluoropyrimidines is understood, the molecular pathway leading to fluoropyrimidine-induced apoptosis is not well characterized; however, p53 and Bax are putative molecules at the apex of this apoptotic cascade ([169](#)).

Two 5-FU resistant derivatives of the human colorectal carcinoma cell line HCT116 were examined and characterized by their genotypic and phenotypic alterations in comparison with the parental cell line ([170](#)). The apoptotic fractions of the resistant variants were approx 50 and 33% lower than the parental cell line. Microarray analysis revealed differential regulation of genes involved in apoptosis, metastasis, proliferation, and DNA repair. Thus, drug resistance may be acquired not only by deregulating a drug’s target enzyme, but also, by interfering with a complex network of apoptotic and metastatic pathways.

In the Dunning rat prostate metastatic cancer model (R-3327 AT3), highly metastatic Bcl-2-transfected AT3 cells were more resistant than vector control AT3 cells to 5-FU-induced apoptosis, and were 40-fold more resistant to 5-FU cytotoxicity ([47](#)). S-1, a more recently developed oral fluoropyrimidine with a therapeutic index higher than 5-FU, caused persistent apoptosis in vivo and reduced tumor burden ([171](#)). Treatment with S-1 statistically reduced the number of peritoneal micro and macrometastases of gastric cancer and prolonged survival until sacrifice ([172](#)). UFT, an oral fluoropyrimidine, was used in combination with the bisphosphonate zoledronic acid in the 4T1/luc murine breast cancer model, which spontaneously disseminates to bone following orthotopic mammary fat pad inoculation ([173](#)). In vivo, this drug regimen suppressed bone metastases without affecting primary tumor growth. In vitro, the combination of 5-FU and zoledronic acid significantly increased the cellular apoptotic fraction ([173](#)). Hence, fluoropyrimidine sensitivity is related to inhibition of metastatic growth, at least in part, through the induction of apoptosis.

5.2. Taxanes

Primary tumors and metastases are differentially sensitive to the taxanes, a class of agents that promote microtubule assembly and stabilize microtubules against depolymerization. The parental taxane paclitaxel (Taxol) and its second generation analog

docetaxel have a broad spectrum of antitumor activity which may be attributed to the continuum of signaling pathways altered in response to these agents. Disruption of microtubule dynamics alters gene expression, apoptotic signaling, angiogenesis, and the EGFR and Ras proliferation signaling pathways (reviewed in ref. 174). In a pharmacodynamic analysis of Taxol in primary and metastatic rat prostate MAT-LyLu tumors, accumulation and retention of Taxol was identical in the primary and metastatic tumors (175). However, primary tumors were consistently more sensitive than metastases to Taxol-induced growth inhibition (175). Thus, differential sensitivity to the taxanes results not from innate pharmacokinetic differences between the primary tumor and its metastases, but rather, from intrinsic differences in the responses of the molecular signaling pathways.

One study, investigating the mechanisms behind Taxol's antimetastatic effects, revealed Taxol-induced alterations in gene expression that were involved in angiogenesis and metastasis. In the C57BL/6 mouse melanoma model, Taxol failed to affect primary tumor size but inhibited spontaneous lung metastases (176). Taxol induced expression of *E-cadherin* and the metastasis suppressor *Nm23*, while downregulating *VEGF* expression. Not surprisingly, Taxol-treated cells underwent significant apoptosis. These data describe an intricate interplay between microtubule disruption and pathways of angiogenesis, metastasis, and apoptosis.

Where taxanes are incapable of inducing apoptosis, resistance and metastatic outgrowth emerge. Treatment with antimicrotubule agents like the taxanes leads to phosphorylation of Bcl-2 and Bcl- x_L , which inhibits their mitochondrial membrane channel function and subsequent antiapoptotic activity (Table 1) (174). When non-metastatic breast carcinoma MCF-7 or MDA-MB-468 cells were transfected with either *Bcl-2* or *Bcl-x_L*, Taxol resistance was elevated approximately 100-fold (16). Similarly, primary cultures of *Bcl-x_L*-transfected MDA-MB-435 breast cancer cells established from lung, lymph node, or bone metastases showed almost twofold greater resistance to docetaxel (56). Therefore high *Bcl-2* and *Bcl-x_L* expression, which support apoptosis resistance, anoikis resistance, and genomic instability (as outlined earlier in this chapter), is not only conducive to metastatic growth, but also challenges the pro-apoptotic effects of the taxanes.

5.3. Nitric Oxide

There is considerable controversy regarding the effects of nitric oxide (NO) and nitric oxide inhibitors on tumor cell metastasis. NO has pleiotropic effects on pathways known to contribute to metastasis; namely angiogenesis, migration, and apoptosis. When expressed at low levels, NO mediates vasodilation, smooth muscle relaxation, neurotransmission, and inhibition of platelet aggregation; when induced to higher levels, NO can contribute to inflammation and carcinogenesis (177). Production of NO is catalyzed by a family of enzymes, the nitric oxide synthases (NOS), of which there are three characterized isoforms: NOSI (nNOS) and NOSIII (eNOS) are constitutively expressed neuronal and endothelial isoforms, respectively, whereas NOSII (iNOS) can be induced upon exposure to inflammatory cytokines.

NO production triggers p53-mediated apoptosis via induction of DNA damage and PARP cleavage (177). Although this would imply a straightforward correlation between high NO expression, apoptosis, and therefore, inhibition of metastasis, the actual role of

Table 1
Potential Therapeutic Molecular Targets in Apoptosis and Metastasis

Gene	Function	Therapeutic intervention	Resultant effect to stimulate apoptosis
Bcl-2; Bcl-x _L	Mitochondrial membrane permeability	Bcl-2 antisense, Taxanes	Phosphorylation of Bcl-2/ Bcl-x _L and subsequent inhibition of mitochondrial membrane channel function
Bax	Mitochondrial membrane permeability	5-fluorouracil, gene therapy	Elevation of expression
NF-κB	Transcription factor	Proteasome inhibitors (PS-341)	Blockade of IκB degradation, which maintains NF-κB in an inactive state
p53	Transcription factor	DNA damaging agents (ionizing radiation, adriamycin), antimetabolites (5-fluorouracil), proteasome inhibitors (PS-341), cyclin dependent kinase inhibitors (flavopiridol), gene therapy	Activation of p53 by increasing its stability and expression
DAPK	Calcium/calmodulin serine/threonine kinase	De-methylating agents (5-Aza C)	Reversal of promoter methylation and subsequent gene activation
Maspin TRAIL	Serine protease inhibitor TNF family ligand involved in extrinsic apoptotic pathway	Gene therapy, DR monoclonal antibodies, TRAIL agonists	Restoration of expression; tumor cell-specific apoptosis

NO in metastasis is unclear. NO sensitivity to apoptosis and metastasis is dependent on several factors: NO source (host tissue versus tumor cell), cell context (genetic constitution, cell type, p53 status), and NOS isoform expression ([178](#)).

Murine melanoma K-1735 cells and their high, low, and nonmetastatic clones were exposed to various NO-inducing cytokines and cytotoxicity was examined ([179](#)). The low and nonmetastatic clones were highly sensitive and produced significant levels of NO upon cytokine induction. In contrast, the highly metastatic clones did not produce NO to any discernible degree and, accordingly, had lower NOS activity than the non-metastatic cells. These in vitro experiments suggested an inverse correlation between NOSII activity and metastatic potential. Similar conclusions were reached in clonal populations of murine pancreatic adenocarcinoma cells (Panc02), in which high *NOSII* expressing clones failed to produce primary tumors and metastases, whereas low-*NOSII* expressers were tumorigenic and produced liver metastases and ascites ([180](#)). Confounding these two studies are the results from Jadeski et al. ([181](#)) who observed

the converse, that is, highly metastatic clones of the murine mammary carcinoma C3H/HeJ model expressed high levels of *NOSIII* and *NOSII*, whereas the weakly metastatic clones expressed low levels of *NOSIII*. Whether these differences were cell context specific or caused by specificity of NO-producing isoforms remains unclear.

Another significant factor underlying NO sensitivity and metastasis is the effect of host-derived NO production. When the *NOSII*^{-/-} KX-dw cell line was injected into syngeneic *NOSII*^{+/+} and *NOSII*^{-/-} C57BL/6 mice, tumors from *NOSII*^{+/+} mice had high *NOSII* expression and activity and produced few lung metastases (182). On the contrary, tumors in *NOSII*^{-/-} mice had undetectable *NOSII* expression and a significant number of pulmonary metastatic nodules. The authors concluded that host physiological *NOSII* expression directly inhibited tumor growth and metastasis. However, although host derived *NOSII* may differentially modulate metastatic progression, tumor cell NO sensitivity and production play an equally important role. In *NOSII*^{+/+} or *NOSII*^{-/-} syngeneic mice, B16 melanoma cells were highly resistant to NO-induced cytotoxicity whereas the murine ovarian carcinoma M5076 cells remained sensitive (183). Despite no differences in primary tumor size, B16 cells produced more metastases in *NOS*^{+/+} mice than in *NOS*^{-/-} mice, and M5076 cells produced fewer metastases in *NOS*^{+/+} mice than *NOS*^{-/-} mice.

Just as the in vitro and in vivo evidence regarding the role played by NO in tumor cell metastasis is contradictory, so too is the clinical data. High total NOS expression corresponded to improved survival in lung carcinoma patients (184). When analysis was conducted separately on the three NOS isoforms, high-NOSII staining was associated with lower grade lung carcinoma (184). However, compelling but directly contrasting evidence suggested that high-*NOSII* mRNA or protein levels were positively correlated with lymph node metastases in oral squamous cell carcinoma, breast cancer, and head and neck cancer (reviewed ref. 177). Here again, differences may result from tumor cell type specificity. What is questionable then, is the efficacy of NO inhibitors as therapeutic agents, given the complexity of tumor–host interactions. Based upon the above evidence, it seems a better understanding of NO's promotion or inhibition of metastasis is required prior to launching NO intervention into the clinical setting.

6. Overcoming Apoptotic Resistance

Conventional therapeutic approaches in the treatment of cancer typically induce apoptosis. We have established that metastatic tumor cells have evolved a multitude of mechanisms to evade apoptosis by either directly disrupting apoptotic pathways, downstream targets of these pathways, or the actual apoptotic machinery. Many metastases are thus refractory to traditional therapeutic approaches and this explains why metastasis is so fatal. Effective treatment strategies will thus rely on overcoming resistance to apoptosis.

6.1. Paradigm for Diagnosing and Treating Apoptotic Resistance

Bold et al. (185) offer the following paradigm for tackling this resistance issue: upon biopsy, the apoptotic responsiveness of the tumor should be assessed by determining its apoptotic index and the expression levels of p53, Bcl-2 family members and other molecular components of programmed cell death. If the tumor is responsive to apoptotic stimuli, then a traditional course of chemotherapy and ionizing radiation may be followed. However, if the tumor has a resistant phenotype, additional steps must be taken to restore

sensitivity to apoptotic stimuli. These additional steps include gene therapy and/or a combination of gene therapy/chemotherapy, which will be discussed in brief below.

6.2. Restoring Sensitivity

Restoring sensitivity to apoptotic stimuli involves either restoring expression of key regulators, artificially supplying cancer cells with the critical gene involved in apoptosis, or inhibiting prosurvival pathways active in metastatic cells. Of course, in order to restore function, the aberrant gene/protein must first be identified. Table 1 summarizes the therapeutic approaches described in this section that are currently being used or may potentially be used to conquer apoptotic resistance in cancer.

Effective drugs have been identified to stimulate re-expression of particular apoptotic regulators. Several genes involved in apoptosis have reduced expression in metastatic cells as a result of hypermethylation of their promoters, including *Apaf-1*, *maspin* and *DAP* kinases (40,41,43,75–77). Therefore, combination chemotherapy with such de-methylating agents as 5-aza C may restore cytotoxicity of apoptosis-inducing compounds. In metastatic lesions with functional, wild-type p53, induction of p53 expression with such DNA-damaging agents as adriamycin may repress cell survival pathways thereby prompting cell death when administered in addition to apoptosis-inducing drugs. For cancers harboring mutations in p53's DNA-binding region, pharmacological compounds that stabilize the DNA-binding domain may also hold promise in a combination chemotherapeutic course with cytotoxic drugs (186).

Gene therapy and/or administration of recombinant proteins for apoptotic regulators that are not functional in metastatic cells are additional approaches to restore sensitivity to cytotoxic drugs. Several groups, including Introgen Therapeutics and Onyx Pharmaceuticals, are attempting p53 gene therapeutic approaches with adenoviral-based vectors. In lung cancer, viral delivery of wild-type p53 substantially enhanced the apoptotic index, which may be further augmented by cytotoxic drug therapy and/or radiotherapy (187). TRAIL is a particularly intriguing candidate for gene therapy given its propensity to induce apoptosis specifically in tumor cells. Administration of adenoviral vectors expressing TRAIL or recombinant TRAIL protein delivered by liposomes has shown much potential in experimental animal models (108,109,188). In a mouse model of breast cancer, TRAIL gene therapy in combination with the chemotherapeutic agent paclitaxel synergistically reduced lung metastases (110). Other potential apoptotic regulators that may be appropriate for this kind of gene replacement therapy include *Bax* (currently in phase I clinical trial) (189), *maspin* and the *DAP* kinases. It should be added, however, that the efficacy of any of these gene therapeutic approaches depends on targeted gene delivery specifically to sites of tumor growth.

TRAIL-induced apoptosis might also be triggered in cancers with a monoclonal agonist antibody to the TRAIL receptors. One such agonist antibody against DR4 is currently in a phase I trial (Human Genome Sciences) and a similar recombinant antibody exhibited potent antitumor effects in a mouse model (190). Of course, this therapeutic approach relies on DR expression. Tumor cells that have developed TRAIL resistance by diminished DR expression or decoy receptor expression will not be amenable to agonist-based therapy.

The latest approach in treating apoptotic resistance involves antisense technology. Short synthetic RNA or DNA oligonucleotides with sequences complementing the mRNA sequence of specific genes are delivered to tumor cells to interfere with the

translation of the coded protein. This approach would be utilized to disrupt expression of genes that function to suppress apoptosis, such as *Bcl-2*, *Bcl-x_L* and *survivin*. In fact, antisense *Bcl-2* therapy (Genasense[®], Genta, Inc.) in combination with chemotherapeutic agents such as paclitaxel, cyclophosphamide, and irinotecan is in phase II and III clinical trials for the treatment of several types of cancer including prostate and melanoma (reviewed by ref. 189). But again, the efficacy of this therapeutic approach depends on targeted delivery of the disruptive oligos to the tumor.

Drug-mediated inhibition of apoptotic inhibitors may also enhance the cytotoxic effects of traditional chemotherapy. Proteasome inhibitors, such as PS-341, have potential in overcoming apoptotic resistance by increasing the stability of a number of apoptotic promoters, including p53 and the NF- κ B inhibitor I κ B, and triggering the SAP kinase JNK (191). Suppression of signaling through the neurotrophic receptors, which block anoikis, with the pan-Trk inhibitor CEP-701 has some potential in relieving cell survival signals and reducing metastases in prostate cancer models (162,165). Finally, several potential small-molecule antagonists of Bcl-2 are under investigation, including GX-01 from Gemin-X and the natural product Tetrocarcin-A (Kyowa-Hakko Kogyo) (reviewed ref. 189). As technology improves and drug discovery progresses, additional more specific and efficacious modes of therapy will become available for the treatment of apoptotic resistance and metastatic disease.

7. Conclusion

We are moving into the era of tailor-designed therapeutics based on molecular diagnosis. Much progress has been made elucidating the molecular mechanisms of cell death resistance in metastatic progression. Given the sheer number of potential molecular players that may contribute to this resistance, we need to develop more sensitive and comprehensive diagnostics for determining the faulty gene/genes responsible for apoptotic resistance, in order to best design a course of therapy to overcome it.

In light of the emerging field of cancer stem cell research, we must also consider what population of tumor cells should be assessed for faulty gene function in a clinical diagnosis. Much of the data regarding apoptotic resistance in metastasis is derived from tumor cohort studies, which generally consider tumors as clonal entities, in particular those studies that use immunoblotting and RT-PCR to measure gene expression. However, it should be stressed that tumors consist of heterogeneous populations of cells. Recently, cancer stem cells have been isolated from solid tumors and it is these cells that are believed to be responsible for tumor generation and/or regeneration, in the case of metastasis (192–195). Cancer stem cells, like normal stem cells have the capacity for self-renewal and the ability to undergo a broad range of differentiation events. Unlike normal tissue stem cells, however, cancer stem cells do not have restricted proliferation (reviewed in refs. 194,196). Thus far, information pertaining to the behavior of cancer stem cells is limited, but some evidence implies that they are both resistant to anoikis and resistant to some cytotoxic drugs (193,197). This newly emerging model of cancer development via cancer stem cells would suggest that both diagnostic and therapeutic efforts should be focused on cancer stem cells to diminish disease recurrence and spread.

Efforts should also be focused on the persistent need for more effective therapies to overcome apoptotic resistance in metastasis. Gene and antisense therapy are clearly limited by delivery mechanisms. Small molecule, rational and *in silico* drug design to

the key molecular targets that have already been identified may provide us with an improved species of chemotherapeutics for attacking metastases, or more specifically the cancer stem cells of metastatic lesions.

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Carcinogenesis

Balance Between Apoptosis and Survival Pathways

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Summary

Numerous molecules and pathways have been identified that control various aspects of apoptosis and survival signaling. A dynamic balance between these opposing activities is required to fine-tune biological systems under both normal and stressed conditions. In this chapter, pathways leading to apoptosis are briefly summarized. This is followed by a discussion of various survival pathways that are activated by both prosurvival and proapoptotic stimuli. Finally, a model depicting the simultaneous engagement of both pro-survival and pro-death pathways by stress signals is presented.

Key Words: Apoptosis; survival; stress; NF- κ B, reactive oxygen species (ROS); FOXO3a; lipocalin.

1. Introduction

Apoptosis, a form of cell death involving a series of well-organized events requiring active cell participation, is the basis for normal tissue remodeling as well as the end result of certain toxic insults (*1*). Numerous molecules and pathways control various aspects of apoptosis signaling (*1,2*), and the fine-tuning of biological functions often requires a dynamic balance between opposing activities. This is most obvious in the nervous and immune systems, but a similar balance between death and survival signals appears to exist and to be essential for normal tissue development and homeostasis. Similarly, a balance among pro- and antiapoptotic factors determines the fate of a cell being stressed by stimuli including xenobiotics (*Fig. 1*).

The expression or functions of genes controlling both apoptotic and survival pathways are often altered in cancer cells. These changes allow the cells to escape apoptotic signals and proliferate indefinitely. Disrupting the balance of cellular decisions regarding life and death is thus a critical factor in the progression and in the treatment of cancer (*2,3*). Because most cancer therapies act primarily by inducing apoptosis, the resistance of cancer cells to death has serious clinical implications.

2. Extrinsic and Intrinsic Apoptotic Pathways

Apoptosis, which is the default signal for many cells, is normally suppressed by survival signals that are generated both internally and through molecules derived from neighboring cells. Apoptosis may be initiated by removing or blocking essential survival signals, or in response to the more direct activation of the extrinsic (death

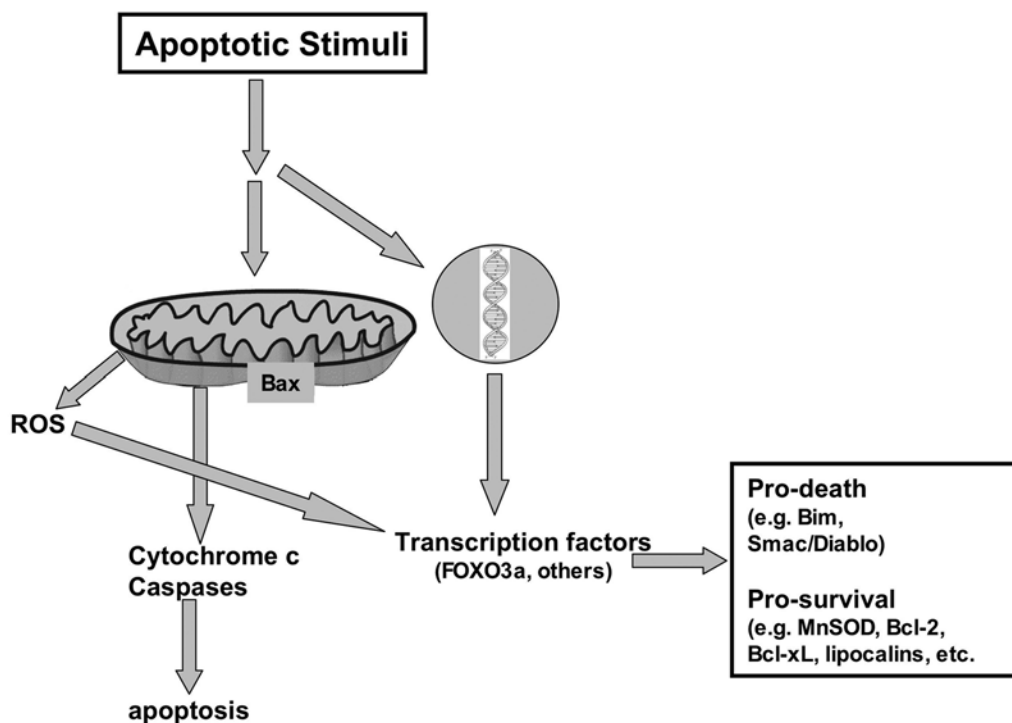


Fig. 1. Schematic depicting the balance among pro- and antiapoptotic factors which determine the fate of a cell being stressed by apoptotic stimuli.

receptor) or intrinsic (mitochondrial) death pathways (*1,4*). Both pathways converge on the initiator and effector caspases. These enzymes carry out a proteolytic cascade that dismantles cellular structures resulting in the final demise of the cell. At the same time, however, prosurvival pathways may be activated.

All caspases are synthesized as inactive procaspases that become activated by autocatalysis or cleavage by other caspases. There are two groups within the caspase family; initiator and effector caspases (*5,6*). Initiator caspases have long prodomains that contain a protein–protein interaction platform for the recruitment of procaspases into activating protein complexes including the death-inducing signaling complex (DISC) and the apoptosome. Initiator caspases such as caspase-8 and -10 have a death effector domain (DED) whereas caspase-1, -2, -4, -5, -9, -11, and -12 have a caspase-activating recruitment domain (CARD). Effector caspases lack the long N-terminal nonenzymatic prodomain. These caspases include caspase-3, -6, and -7. They are also known as the executioner caspases because they are responsible for the physiological manifestations of apoptosis.

In the extrinsic death pathway, activation of the death receptors by external ligands recruits initiator caspases into the DISC. Major death receptors include Fas (CD95), tumor necrosis factor (TNF) receptor, DR3 (Apo-3), DR4 (TRAIL-R1), and DR5 (TRAIL-R2) (*7*). Once these death receptors are stimulated by their respective ligands, components of the DISC machinery are recruited through interaction of the death domains (DD). Fas-associated death domain (FADD) is a common factor that functions

as a critical adaptor protein to recruit caspase-8 and -10 through its DED. FADD associates directly with Fas, DR4 and DR5 receptors, but binds to the TNF receptor through association with the TNF receptor 1-associated death domain (TRADD). The proximity of caspases-8 and -10 in the DISC results in their activation presumably by an allosteric mechanism (8–11). These initiator caspases, once activated, can then cleave and activate downstream effector caspases.

The intrinsic apoptotic pathway works through mitochondria, which contribute to apoptosis in at least three ways: (1) the release of pro-apoptotic molecules, (2) increased production of reactive oxygen species (ROS), and (3) impaired ATP production. The release of apoptotic mediators from mitochondria is a critical event in the intrinsic pathway. Cytochrome *c* is one of the key factors released (12). Upon its release, cytochrome *c* promotes the formation of a proapoptotic complex called the apoptosome. This complex is comprised of apoptotic protease-activating factor-1 (Apaf-1), caspase-9, and ATP. In this complex, caspase-9 is activated (8,13) and is then able to cleave downstream effector caspases like caspase-3.

The ability of the activated caspases to initiate apoptosis is further regulated by the inhibitor of apoptosis proteins (IAPs). IAPs (especially XIAP) inhibit both activated caspase-9 and -3 (13). In addition, XIAP and IAP1/2 have a carboxyl-terminal motif found in RING-finger proteins that allows them to function as ubiquitin ligases promoting the proteasomal degradation of bound caspases (14). To counter this, the mitochondria release, in addition to cytochrome *c*, other proapoptotic factors including second mitochondria-derived activator of caspases (Smac/DIABLO) and Omi/HtrA2. These factors bind to and sequester XIAP allowing activated caspase-3 to function (15–17). Other proapoptotic mitochondrial intermembrane space proteins that are released during apoptosis include the apoptosis-inducing factor (AIF) and endonuclease G. These factors cause apoptosis independent of caspases. AIF translocates to the nucleus and causes chromatin condensation and large-scale DNA fragmentation while EndoG translocates into the nucleus and helps digest nuclear DNA (18,19).

The mitochondrial production of ROS, and the loss of mitochondrial membrane potential, may contribute to apoptosis. When the membrane potential is lost, ATP is not synthesized and ROS accumulate (20). Loss of the mitochondrial membrane potential can be caused by a permeability transition pore (PTP) which contains the inner membrane protein, adenine nucleoside translocator (ANT) and the outer membrane protein, voltage dependent anion channel (VDAC) (21,22). The PTP pore has been implicated in the release of mitochondrial factors upon apoptotic stimuli. In addition, several members of the Bcl-2 family including Bax and Bak are involved in the apoptosis signaling caused by changes in the PTP (for reviews, see refs. 23–25).

The role of ROS in apoptosis is still not clearly understood mechanistically. There is evidence that antioxidants can abolish the apoptotic response of many diverse stimuli. Thus, ROS may be an important mediator of apoptosis (26). In addition, anti-apoptotic Bcl-2 family members decrease ROS production in response to apoptotic stimuli (27,28). Recent evidence highlights a specific role of ROS in the control of redox balance required for proliferation signaling. This includes the activation of the transcriptional factor activator protein-1 (AP-1) through the mitogen-activated protein kinase (MAPK) family (reviewed in ref. 29). This ultimately leads to the transcriptional upregulation of genes involved in cellular proliferation. Thus, ROS play a dual

role in mediating apoptosis as well as mitogen- or survival factor-induced cell proliferation and survival (*see Subheading 3.4.*).

Crosstalk between the extrinsic and intrinsic apoptosis pathways exists. This occurs mainly through cleavage of the Bcl-2 family member, Bid by active caspase-8 into a truncated form t-Bid. Once cleaved, t-Bid translocates to the mitochondria and either directly binds to and induces the oligomerization of Bax, or binds to and inactivates pro-survival Bcl-2 family proteins like Bcl-2 and Bcl-xL (30). Because caspase-8 is the common target for DISC activation through the DRs, Bid therefore bridges the extrinsic and intrinsic pathway.

3. Survival Pathways

3.1. Pro- and Antiapoptotic Bcl-2 Proteins

The proapoptotic Bcl-2 proteins, Bax and Bak, function as gatekeepers to the release of apoptotic factors from the mitochondria. Opposing their activities are the gatekeeper, antiapoptotic Bcl-2 family proteins. The antiapoptotic Bcl-2 family members, including Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Boo and Bcl-B, share sequence conservation in all four BH domains (i.e., BH1 to BH4). In contrast, the proapoptotic Bcl-2 proteins, Bax, Bak and Bok, share sequence similarity in only the first three BH domains. The BH3-only proteins, which include Bid, Bik, Bim, Bad, Bnip3, Blk, Bmf, Hrk, Noxa and Puma, exist in inactive forms during normal conditions and become activated or induced in response to apoptotic stimulation. The balance of these pro- and antiapoptotic Bcl-2 proteins contributes to the survival or death of a cell.

There are several models of how antiapoptotic Bcl-2 family proteins promote cell survival. One involves binding of the anti-antiapoptotic proteins to the BH3-only proteins preventing them from activating the gateway proteins Bax, Bak, or they can directly bind to Bax or Bak preventing their activation (31,32). Other potential models involve Bcl-2 preventing the intracellular calcium flux, pH and ionic changes that occur early during apoptosis (33,34), or binding to and inhibiting a membrane bound protein X that is an activator of Bax and Bak oligomerization (35,36).

There are four mechanisms by which antiapoptotic Bcl-2 proteins can function and contribute to carcinogenesis. The first involves their subcellular localization. Bcl-2 and Bcl-xL are found on the mitochondrial outer membrane where they can inhibit the oligomerization of Bax and Bak (37). Bcl-2 and Bcl-xL also localize on the ER membrane where they may affect calcium uptake or release during apoptosis (38). In addition, Bcl-2 localizes in the nuclear membrane and may modulate nuclear trafficking of transcription factors such as NF- κ B, AP1, CRE, and NFAT (39). The second mechanism involves levels of expression. Overexpression of Bcl-2 family proteins confers resistance to many apoptotic stimuli (40,41). In addition, the antiapoptotic Bcl-2 proteins are often overexpressed in cancer cells. In fact, Bcl-2 was discovered as an overexpressed gene in follicular lymphomas as a result of a translocation of the gene from chromosome 14;q32 to chromosome 18;q21 (42). The third mechanism involves phosphorylation (43). For example, phosphorylation of Bcl-2 at serine residues in some cell systems results in the loss of its antiapoptotic functions (44,45) whereas phosphorylation at serine 70 appears necessary for its antiapoptotic functions (46). Other studies show that phosphorylation in the unstructured loop domain of Bcl-xL and Bcl-2 diminishes their

antiapoptotic activities (47). Finally, posttranslational mechanisms can affect Bcl-2 family members functions. For example, cleavage of Bcl-2 by caspase-3 results in a truncated protein with proapoptotic activity (48). This occurs late during apoptosis thus playing an amplification role.

3.2. The NF- κ B Pathway

The NF- κ B transcription factor is a classic example of a survival pathway activated by apoptotic stimuli. NF- κ B is normally sequestered in the cytoplasm by I κ Bs (inhibitors of NF- κ B). When NF- κ B is released from the I κ Bs, it translocates into the nucleus, binds to DNA, and transcriptionally activates various antiapoptotic genes including IAP1/2, XIAP, Bcl-xL, caspase-8-FLICE inhibitory protein (c-FLIP), and Traf1/2 (49,50). These factors work together to block apoptosis at multiple steps along the apoptotic cascade. For instance, in the Fas receptor pathway, c-FLIP upregulated by NF- κ B interacts with FADD and procaspase-8 to prevent the activation of procaspase-8 (51). IAPs bind to and inhibit the activities of activated caspases (52). TRAF1/2 are adaptor proteins involved in the TNF receptor signaling pathway (53).

In order to overcome the antiapoptotic function of NF- κ B, several key components of this pathway, including RelA, can be degraded by caspases (54). Caspase-3 also cleaves I κ B α creating a super repressor of NF- κ B (55). The IKK complex can be inactivated by caspases (56). Lastly, receptor-interacting protein (RIP) and TRAF2, both of which are involved in NF- κ B activation, are also substrates of caspases (57,58). Thus, the NF- κ B pathway is a complex network integrating both pro- and antiapoptotic signals upstream and downstream of this transcription factor.

3.3. The PI3K/Akt Pathway

Another complex survival network involves phosphatidylinositol 3 kinase (PI3K) and Akt. Akt is a serine/threonine kinase first identified in humans as two genes homologous to the viral oncogene *v-akt* that causes a form of mouse leukemia (59). Additional studies showed that these homologues coded for a kinase similar to protein kinase C (PKC), leading to the alternate name of protein kinase B (PKB). There are now three members of this family, referred to as Akt1,2,3 (or PKB α,β,γ) (60). The three genes show over 80% homology at the amino acid level and have a rather broad distribution. Recently, knockouts of each isoform have been generated (reviewed in ref. 61). These knockouts have shown diverse phenotypes suggesting distinct regulatory roles for the isoforms. However, all individual knockouts are viable, whereas a double Akt1-Akt2 knockout is lethal shortly after birth (61). This indicates functional redundancy among these proteins.

Akt is activated through a dual process involving translocation to the plasma membrane and phosphorylation at Ser473 in the C-terminus and Thr308 in the activation loop. The phosphorylation at Thr308 by PDK1 is considered essential because mutation to an alanine moiety, which cannot be phosphorylated, greatly diminishes Akt activity (62). Akt phosphorylation involves the generation of phosphatidylinositol-3,4,5-trisphosphate (PIP₃) from phosphatidylinositol-4,5-bisphosphate (PIP₂) by phosphoinositide 3-kinase (PI3K). Binding of the pleckstrin homology domain of Akt to PIP₃ relocalizes the cytoplasmic Akt protein to the plasma membrane, allowing PDK1 to phosphorylate Akt at Thr308. The mechanism of Ser473 phosphorylation is unclear. Both autophosphorylation

and phosphorylation by several distinct kinases (one called PDK2) have been implicated (63,64). A further important regulatory feature of Akt activation is PTEN, a phosphatase whose main physiological substrate is PIP_3 . PTEN thus functions as a negative regulator of PI3K signaling.

A number of kinases contain the consensus sequence for activation by PDK1 (65). Thus, whereas Akt has received the most attention, other downstream kinases may also be affected when PDK1 activity is altered. One of significant interest is serum and glucocorticoid regulated protein kinase (SGK), which has 54% sequence identity in the catalytic domain with Akt (66). SGK differs from Akt by lacking the pleckstrin homology domain and is thus activated in the cytosol rather than after membrane binding. In addition, the consensus sequence phosphorylated by SGK differs from that of Akt (67). This, and some other differences related to the hydrophobic amino acid composition of peptide substrates, suggests that SGK and Akt have complementary rather than redundant functions (67). Nevertheless, like Akt, SGK is induced by several stimuli including injury, suggesting it has a role in cell survival.

Akt activates various downstream targets that regulate apoptosis, cell cycle, DNA repair, nitric oxide production, and glycogen metabolism (68). Akt protects the cell from apoptosis by phosphorylating various apoptotic regulators such as Bad, caspase-9 and FKHR1 (Forkhead receptor-1). Akt phosphorylation of Bad induces its interaction with 14-3-3 protein, which causes a conformational change in Bad leading to its phosphorylation by protein kinase A. This disrupts the ability of Bad to bind to Bcl-2 and Bcl-xL thus freeing these factors and allowing them to inhibit apoptosis (69). Akt phosphorylation of FKHR1 also leads to its binding to 14-3-3 proteins in the cytosol thus preventing FKHR1 from translocating to the nucleus. This prevents FKHR1 from activating proapoptotic genes such as Bim and FasL (70). Similarly, Akt phosphorylation of procaspase-9 prevents its activation by the apoptosome (71). Akt is also an indirect negative regulator of p53. It phosphorylates Mdm2, increasing its ability to translocate into the nucleus where it binds to p53 and promotes its degradation (72). Lastly, Akt can activate the NF- κ B pathway by phosphorylating IKK α leading to the phosphorylation and degradation of I κ B (73,74). Therefore, Akt can promote cell survival by antagonizing both the extrinsic and intrinsic apoptotic pathways.

3.4. The MAPK/ERK Pathway

Extracellular regulated kinase (ERK) is a member of the MAPK family. Activation of ERK generally protects a cell from apoptosis through the phosphorylation and activation of downstream transcription factors that regulate antiapoptotic molecules such as IAPs, TRAF1/2 and Bcl-xL (75). The Raf/MEK/ERK pathway also affects cell-cycle regulatory proteins, thereby regulating cell-cycle progression. This pathway is activated by extracellular signals that recruit the adaptor protein Grb2 (growth factor receptor bound protein 2). This protein then binds to the cytoplasmic side of cell surface receptors like EGFR and PDGFR through its Src homology 2 domain (76). Grb2 is constitutively bound to son of sevenless (SOS), a guanine nucleotide exchange factor that, following activation, exchanges the GDP for GTP on RAS (77). Upon Grb2 recruitment to the plasma membrane SOS gets activated (77). This causes a conformational change in RAS allowing it to bind to a MAPK, RAF1. RAF1 is activated by binding to RAS and phosphorylates the MAPK, MEK1 and MEK2. These kinases in turn phosphorylate

ERK1 and ERK2. Following activation, ERKs translocate into the nucleus and phosphorylate a variety of substrates. These include the 90 kDa ribosomal S6 protein kinase (p90^{rsk}), the cytosolic phosphatase A2, and several transcription factors like NF- κ B, c-Myc, Ets, CREB and AP-1 (75).

The contributions of different MAPK family members to apoptosis have been examined following withdrawal of NGF from rat PC-12 pheochromocytoma cells (78). They showed that this led to the sustained activation of JNK and p38-MAPK and the inhibition of ERKs. The combined effects of dominant negative as well as constitutively activated forms of various factors in these three pathways show that the simultaneous activation of JNK and p38MAPK and the inhibition of ERK is needed for induction of apoptosis in these cells (78). This implies that ERK is a survival factor that needs to be silenced in order for apoptosis to occur.

Recently, multiple studies have revealed a more direct prosurvival mechanism of the ERK pathway. Ras/Raf/MAPK/ERK activation by, serum, endothelial growth factor (EGF), platelet-derived growth factor (PDGF), insulin, integrin-mediated adhesion, or thrombin, results in the phosphorylation of a critical BH3-only protein, Bim (79–86). Phosphorylation of Bim either inhibits its interaction with Bax (79) or leads to proteasome-dependent degradation (85).

4. Cell Survival Signaling During Apoptosis

Although great progress has been made in understanding the details of apoptosis pathways, little is known about how cells initially respond to apoptotic stimuli. For example, when a population of cycling cells is stimulated by an apoptotic signal, do they immediately enter the apoptotic mode or do they first stage a defensive response? Alternately, do the stimulated cells simultaneously activate both prosurvival and prodeath mechanisms? Is it then the balance between survival and death signals that ultimately determines when and whether the stimulated cells will die? Or is it possible that an adaptive response is activated that helps prevent excess cell death? This would explain the observation that in the presence of apoptotic stimuli, surviving cells become more resistant to apoptosis (87). The work described below sheds some light on these questions.

4.1. Prosurvival Mechanisms Induced by Apoptotic Stimuli

Many stressors cause an early mitochondrial activation characterized by a rapid induction of respiration-related proteins including apocytochrome *c* and cytochrome *c* oxidase. These proteins are then rapidly imported into the mitochondria to enhance respiration leading to early membrane hyperpolarization, increased oxygen consumption, and maintenance of ATP levels (88,89). These responses precede the release of holo-cytochrome *c* from the mitochondria and the subsequent activation of caspases (89). These observations suggest that cells, upon apoptotic stimulation, rapidly mobilize defensive mechanisms in order to extend their survival.

Many drugs, chemicals, ligands, and conditions that induce apoptosis also induce an early activation of mitochondrial systems characterized by cytochrome *c* upregulation and increased mitochondrial respiration. The upregulation of the mitochondria respiratory chain proteins and increased respiratory activity most likely represent one aspect of the global mitochondrial activation aimed, perhaps, to maintain appropriate ATP levels

critical for various cell activities associated with apoptosis, as well as for cell survival. In addition to maintaining ATP levels, increased apocytochrome *c* in the cytosol and upregulated holocytochrome *c* in the mitochondria also possess antiapoptotic and prosurvival functions (90–92).

Another class of prosurvival molecules induced or activated by many apoptotic stimuli are antiapoptotic Bcl-2 family proteins, in particular, Bcl-2 and/or Bcl-xL (93). In response to DNA damage, trophic factor deprivation, and a mitochondrial toxin, Bcl-2 and/or Bcl-xL are rapidly induced (93). This induction clearly plays a prosurvival role as inhibition of the upregulation of Bcl-2 and/or Bcl-xL using specific siRNAs facilitated cell death (93). Similarly, hypoxic treatment selectively upregulates Bcl-xL leading to generation of death-resistant cells (94). Ultraviolet irradiation eliminates the antiapoptotic Mcl-1 but also induces increased targeting of Bcl-xL to the mitochondria (95). Moreover, various apoptotic stimuli upregulate Bcl-2 protein levels via a mechanism dependent on the internal ribosomal entry site (96). Finally, ultraviolet-A can upregulate the Bcl-xL protein levels by modulating the 3'-untranslated region (97).

The third class of prosurvival molecules is the superoxide dismutases (SODs) that function by removing the ROS superoxide anions (98,99). We found that all apoptotic stimuli tested upregulated the levels of both MnSOD and Cu/ZnSOD at about the same time when proapoptotic Bim is induced (93). MnSOD siRNA blocked the induction of MnSOD and also enhanced apoptosis (93), suggesting that the upregulated MnSOD is serving a prosurvival function.

A fourth class of prosurvival molecules induced by apoptotic stimuli (and stressful stimuli in general) includes various chaperone and cochaperone proteins. Multiple heat-shock proteins (Hsps) are cytoprotective (100–103). Indeed, we have observed that Hsp60 is rapidly upregulated and/or released from the mitochondria to the cytosol in response to apoptotic stimulation (89), presumably to extend cell survival. The mitochondrial Hsp70 interacts with p66^{Shc}, a molecule implicated in determining the cell's lifespan, to extend cell survival in the presence of stress signals (104). Similarly, the bacterial Hsp60 (GroEL) protects epithelial cells from apoptosis induction via activation of the ERK pathway (105).

Cell cycle-arrested cells generally survive better than proliferating cells (106). Thus, although the lower rates of metabolism in resting cells is likely important in their improved survival, cell-cycle inhibitors such as p27^{KIP1} and p21^{WAF-1}, may also represent prosurvival molecules. For example, p21^{WAF-1} is a critical prosurvival factor transactivated by p53 (107,108); overexpression of p21 confers on colon cancer cells resistance to apoptosis induction by chemicals in both p53-dependent and p53-independent manners (109). In contrast, decreased p21 expression sensitizes cells to apoptosis (110,111). Indeed, we have observed an inverse correlation between p21 expression and apoptosis. When LNCaP cells, a p53-wt prostate cancer cell line, are stimulated with γ -irradiation, etoposide, doxorubicin, or taxol, γ -irradiation and etoposide significantly induced p21 protein expression with little cell death whereas doxorubicin and taxol did not upregulate p21 but caused obvious cell death (unpublished observations).

4.2. Secreted Proteins

A role for secreted proteins in how cells respond to apoptotic stimuli has been little studied. However, recent data demonstrate that the lipocalins 24p3/NGAL (neutrophil

gelatinase associated lipocalin) are profoundly induced in response to several proapoptotic xenobiotics (112). As a secreted protein, 24p3/NGAL may facilitate the survival of surrounding cells.

Lipocalins are a diverse family of more than 20 small soluble, and often secreted, proteins. These proteins have a large degree of diversity at the sequence level (only 20% identity), although most share three conserved motifs. These proteins are defined as lipocalins largely on the basis of their three-dimensional structure, which is comprised of a single eight-stranded, continuously hydrogen-bonded anti-parallel β -barrel. The functions of the lipocalins are not well understood. The cavity in the β -barrel is thought to bind and transport a variety of lipophilic substances (113). In addition, they can bind to specific cell-surface receptors and may deliver ligands, including iron, growth, or survival factors to the cell by receptor-mediated endocytosis (114,115). Importantly, lipocalins appear to have roles in cell regulation, both in terms of proliferation and differentiation (116). This has suggested a number of functions related to cancer where elevated expression of lipocalins is often observed (117). Several lipocalins such as α_1 -microglobulin, $\alpha_2\mu$ -globulin, and orsomucoid are expressed in different forms of cancer and it has been proposed that lipocalins mediate cell signaling either by delivering lipophilic ligands such as retinoids and fatty acids, or by inhibiting proteases (117).

24p3 was first identified as an overexpressed gene in SV40 infected primary kidney cells from mice (118). NGAL, the human analog of 24p3, (NGAL and 24p3 are 62% identical in amino acid sequence [119], but only about 20% identical in nucleotide sequence), was first purified from human neutrophils because of its association with gelatinase (120). Although the association with neutrophil gelatinase suggests the potential to modulate this enzyme, various studies have shown this is not true (120).

Several functions of 24p3/NGAL have been identified but precisely which, if any, are important under normal circumstances is unknown. Functions related to cancer have been suggested (117), but overall little is known about the role of 24p3/NGAL in cell signaling, proliferation, and apoptosis. Some data suggested a role in inflammation, whereas other data indicate NGAL has an important role in iron metabolism (121). A number of inducers of this gene have been found including serum, lipopolysaccharide, various growth factors, retinoic acid, glucocorticoids, and phorbol esters (117,119). In addition, we have demonstrated that several xenobiotics that induce apoptosis, are potent inducers of 24p3/NGAL (112, unpublished data).

24p3 is expressed at high levels in the mammary gland and uterus where it is proposed to induce neutrophil apoptosis thereby allowing selected cells to survive involution (122). The work of Divireddy et al. (123) suggested that extracellular 24p3 had biologic activity in terms of stimulating apoptosis in mouse pro-B FL5.12 cells. On the other hand, as a lipocalin, this protein can bind small lipophilic substances and may thus play a role in transport of signaling species, particularly survival/growth factors. Although the ability of secreted 24p3 to induce apoptosis is uncertain, NGAL appears to have a survival function because recombinant NGAL protein was nontoxic to cells, an antibody to NGAL was a highly effective apoptosis inducer, overexpression of NGAL was protective, and underexpression of NGAL enhanced drug-induced apoptosis (unpublished data). The expression of 24p3/NGAL in all tissues examined (119), and the high levels of expression of 24p3/NGAL by various cancers (117) as well as in normal mammary gland, uterus (122), and testes, is also consistent with a survival

function. Finally, the down regulation of *ExFABP*, a secreted lipocalin that binds fatty acids, induces apoptosis (124), and is supportive of a survival function.

4.3. Transcription Factors Involved in Apoptotic Stimuli-Activated Prosurvival Genes

Other prosurvival signaling mechanisms may exist in stressed cells. For example, apoptotic stimulation may result in a rapid phosphorylation of the translation initiation factor-2, leading to the cessation of *de novo* protein synthesis and providing cytoprotection (125). Overall, induction of prosurvival mechanisms by apoptotic stimuli seems to represent a general phenomenon. Indeed, even apoptosis induced by TNF- α and Fas (discussed below) and death kinase PKR (126) is preceded by an early phase of NF- κ B-mediated prosurvival activities. The induction of prosurvival molecules apparently plays a critical role in extending cell survival as prevention of the induction of Bcl-2, Bcl-xL, or MnSOD by apoptotic stimuli accelerates cell death (93).

In the above examples, the pro-survival molecules are induced either simultaneously with or slightly prior to the induction of various prodeath molecules. Our recent work demonstrates that this occurs at the transcriptional level (93), particularly involving master transcription factors such as NF- κ B, FOXO3a, p53, Rb, E2F1, and c-Myc.

NF- κ B is clearly an important transcription factor in mediating the pro-survival signaling in response to inflammatory, apoptotic, and stress stimuli. Although NF- κ B activation on some genetic backgrounds can lead to apoptosis by, for example, stabilizing p53 (127), repressing the induction of antiapoptotic genes (128), or collaborating with the adenine nucleotide translocator (129), most experimental data suggest that increased NF- κ B activity is associated with resistance to apoptosis (130–137). The prosurvival function of NF- κ B is mainly associated with its induction of prosurvival genes such as IAP, Bcl-xL, and FLIP, as well as SNF1/AMP kinase-related kinase (138) and the ferritin heavy chain (139). The prosurvival function of NF- κ B is well illustrated by the response of cells to the TNF- α family proteins (140–146). Two sequential signaling complexes are formed upon TNF- α binding to the TNF receptor 1 (141). The plasma membrane-bound complex I is rapidly formed upon receptor activation and contains TNF receptor 1, adaptor protein TRADD, DD-containing kinase RIP1, and TRAF-2, leading to NF- κ B activation. Then, complex I exits the receptor and forms a different, long-lived complex II. This complex localizes mainly in the cytosol and contains apoptotic proteins FADD, caspase-8, and caspase-10 in addition to TRADD, RIP1, and TRAF-2. The activation of complex II results in cell death (141). Thus, TNF- α induces the complex II-mediated apoptosis only when the complex I-initiated pro-survival signal (i.e., NF- κ B) is not activated.

Recent evidence indicates that CD95 (Fas ligand) and TRAIL, TNF- α family members conventionally thought to be solely proapoptotic, also activate NF- κ B prior to activating the DISC and caspase-8 (140,142–146). This explains why these death ligands do not induce apoptosis in many cancer cells. In fact, most epithelial cancer cells appear to be so-called type II cells (140) and CD95 stimulation of these cells not only fails to kill them but actually promotes cell migration and invasion (146), possibly through NF- κ B activated urokinase-type plasminogen activator and the SNF1/AMP kinase-related kinase (138). Intriguingly, different from the activation of NF- κ B by TNF- α (141), NF- κ B activation by Fas is mediated through FADD, caspase-8, and RIP and is

inhibited by FLIP (144,145). The concomitant prosurvival and prodeath functions of death ligands such as CD95 and TRAIL are not unique. An increasing number of molecules once thought of as proapoptotic only, including the BH3-only proteins Bad (147,148) and BNIP (149), the mitochondrial protease Omi (150), multi-BH protein Bak (151,152), and activated caspases (153,154) have functions unrelated to apoptosis; even pro-survival functions.

FOXO3a has emerged as a critical regulator of cell death and survival. FOXO3a is a mammalian homologue of *Caenorhabditis elegans* DAF-16 and one of the FOXO (Forkhead box, class O) subclass of Forkhead transcription factor family. FOXO3a plays a critical role in coordinating cell survival and death and regulating stress response and longevity (155). The activities of the FOXO proteins are regulated by phosphorylation. ISGK (67) as well as Akt can phosphorylate (and thus inactivate) (18,25) Forkhead in rhabdomyosarcoma (FOXO1), FKHR-Like 1 (FOXO3a), and acute-lymphocytic-leukemia-1 fused gene from chromosome X (FOXO4) (156). Phosphorylation appears to take place in the nucleus causing FOXOs to bind 14-3-3 proteins. The FOXO/14-3-3 complex is exported to the cytoplasm where the inactive FOXO proteins remain bound to 14-3-3 proteins. In the absence of survival signals, cytoplasmic FOXO is dephosphorylated by PTEN (156), causing dissociation from 14-3-3 proteins and allowing nuclear import, which activates gene expression presumably through a highly conserved monomeric DNA-binding domain (157).

There is a general consensus that phosphorylation by Akt is critical in regulating the function of FOXO factors, and as Akt is inactivated, these transcription factors can become dominant (158). Overexpression of FOXO members can activate some proapoptotic genes and induce apoptosis, although these effects seem to be largely restricted to nontransformed hematopoietic cells (156). FOXO transcription factors also have a role in the regulation of some antiapoptotic genes and can control genes that induce cells to enter a more quiescent cell state where protective mechanisms can act (159).

FOXO3a can transcriptionally activate proapoptotic Bim, TRAIL and TRADD and antiapoptotic MnSOD and cyclin-dependent kinase inhibitor p27^{KIP1} (93,155). FOXO3a also inhibits cell-cycle progression by downregulating cyclin D1 (93,155). We found that FOXO3a is involved in directly regulating the apoptotic stimuli activated Bim and MnSOD (93), two molecules that contain the FOXO3a sites in their promoter regions. Interestingly, several other prosurvival molecules including Cu/ZnSOD, Bcl-2, Bcl-xL, and cytochrome *c* also appear to be partially regulated by FOXO3a as their induction is inhibited by downregulating this transcription factor (dominant negative or siRNA) or in FOXO3a^{-/-} fibroblasts (93). Whether FOXO3a directly or indirectly regulates these molecules remains to be determined.

Other transcription factors, either individually or in combination, may also be involved in the transcriptional activation of both pro- and antiapoptotic molecules in response to apoptotic stimuli. For example, p53 transactivates both prosurvival p21^{WAF-1} (107–111) and multiple proapoptotic molecules such as BH3-only proteins (Bid, PUMA, and Noxa), Bax, and procaspases (160,161). p53 may also protect cells from apoptosis by inhibiting JNK activation (162). Rb similarly regulates the transcription of several life-and-death genes (163). E2F1 not only transcriptionally regulates cell cycle-related genes but also cell death genes including Apaf-1 and caspases (164). c-Myc

transcriptionally regulates molecules involved in cell cycle progression, survival, and death (106). Finally, the transcription factor Nrf2 transcriptionally activates pro-survival genes during apoptotic stimulation, in particular, during endoplasmic reticular stress (165,166).

Recent data point to crosstalk between these master transcription factors. For example, activation of NF- κ B can lead to decreased stabilization of p53 and further enhance cell survival (130). On the other hand, p53 activation can lead to phosphorylation of FOXO3a and changes in its subcellular localization resulting in inhibition of FOXO3a transcriptional activity (167). Newly emerging evidence also makes a connection between FOXO3a and NF- κ B. One study suggests that I κ B kinase inhibits FOXO3a through physical interaction and phosphorylation independent of Akt, which promotes FOXO3a proteolysis via the Ub-dependent proteasome pathway (168). Another study suggests that FOXO3a negatively regulates NF- κ B and FOXO3a deficiency results in NF- κ B hyperactivation and T-cell hyperactivity (169). Overall, several transcription factors activated in response to apoptotic or stressful stimuli clearly mediate the activities of both survival and death pathways.

4.4. Activators Upstream of the Transcription Factors

Master transcription factors such as FOXO3a are themselves induced at the transcriptional level by apoptotic stimuli with distinct mechanisms of action in several genetically distinct cell types (93). This suggests that a common mechanism may be operating to mediate the transcriptional activation of these factors. One possible mechanism involves ROS. These reactive species appear to function as critical apical signaling molecules to activate FOXO3a and perhaps other multifunctional transcription factors (93). This concept is supported by several lines of evidence. First, increased ROS are an early event following many different apoptotic stimuli (88,89,93). Second, many of the antiapoptotic molecules induced by proapoptotic stimuli, including cytochrome *c*, Bcl-2, Bcl-xL, and the SODs, are related to, or induced by, oxidative stress. This raises the possibility that these molecules are induced during apoptotic stimulation to protect against further increases in ROS. Third, suppression of ROS by inhibitors or scavengers inhibits apoptotic signal-induced upregulation of FOXO3a as well as its prodeath and prolife targets. Conversely, artificially generated oxidative stress upregulates FOXO3a and its targets (93). Fourth, the function of ROS as signaling molecules that activate multifunctional transcription factors and ultimately determine the life and death of a cell is consistent with the well established dual survival and death functions of ROS (99,170–174). Finally, FOXO3a (175–180), NF- κ B (99,181), and p53 (99,182) are well-known to be regulated by and also respond to oxidative stress.

5. A Model and Its Implications

The findings discussed above support our proposal that proapoptotic stimuli cause an early mitochondrial activation leading to rapid generation of ROS, which activate master transcription factors such as FOXO3a and NF- κ B. These transcription factors, in turn, activate multiple molecular targets with both proapoptotic and prosurvival functions. These targets appear to be activated as soon as the cell senses stress, and the activation is independent of the extent of the stress and whether or not the final outcome is apoptosis (93). The determination of the ultimate outcome for a cell (survival or death)

seems to be a function of the strengths and timings of the different prosurvival and prodeath signals (93). Presumably, the cell can integrate a myriad of signals leading to the final decision of life or death.

This model (*see* ref. 93 and Fig. 8 therein) has several important implications. First, because both prodeath and prosurvival molecules are activated, the sensitivity of any target cells to the induction of apoptosis will be determined by the balance of these two opposing signals. Second, the induction of prosurvival molecules may occur prior to induction of prodeath molecules. Significant cell killing will occur *only when* proapoptotic signals overwhelm the prosurvival signals or when the latter are eliminated. This prediction is consistent with the recent demonstration that apoptosis elicited by TNF- α proceeds in two steps: an early step, where prosurvival signaling mediated by NF- κ B dominates, and a later step, where prodeath signaling mediated by caspase-8/10 dominates. Cell death occurs only when step two is activated or when step one is inactivated (141). Third, because cells respond to apoptotic stimulation asynchronously and differently, some cells may preferentially upregulate prosurvival molecules, rendering them relatively resistant to further apoptotic stimulation, as often observed in therapy-resistant cancer cells. Similarly, such resistant cells may secrete molecules that protect surrounding cells. Finally, these observations suggest that simultaneously promoting apoptosis and suppressing prosurvival mechanisms will yield the most effective anticancer therapies (183,184).

Acknowledgments

This work was supported in part by NIH grants CA90297 and AG023374, ACS grant RSG MGO-105961, DOD grant DAMD17-03-1-0137 (DGT), and CA83701 (JPK). Support from NIEHS Center Grant ES07784 is also acknowledged.

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Aberrations of DNA Damage in Checkpoints in Cancer

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Summary

Mutations in gene products controlling DNA damage checkpoints and repair pathways cause pre-disposition to a large number of sporadic cancers, hereditary cancer syndromes, and developmental defects. This underscores the vital need for the fidelity of checkpoint control and efficiency for the repair machineries. The checkpoint functions are ensured by multiple, often parallel, pathways and show specificity regarding the nature of the damage, cell-cycle phase, and the subsequent cellular response. The checkpoint control mechanisms also link to other cellular responses such as apoptosis to initiate a death program in the event of unsuccessful repair. It is striking that several checkpoint mutations are associated with developmental abnormalities and cancer syndromes, such as the Nijmegen breakage syndrome and Fanconi anemia, indicating that the maintenance of the genome integrity is essential throughout development. Though several critical DNA maintenance proteins have been identified and their links to tumor progression have been established, alterations of several known checkpoint-associated proteins (e.g., 53BP1, Mdc1, SMC1) in cancer are still undiscovered. Knowledge of the DNA damage checkpoint pathways and pathways sensing the damage and instigating repair will pave the way to improved diagnostics, identification of genetic susceptibility, and, in future, rational therapy of cancer.

Key Words: Checkpoint; cell cycle; DNA damage; p53; H2AX; hereditary cancer syndrome; repair.

1. Introduction

DNA lesions occur as inherent mistakes in the metabolism of DNA, typically during replication, or by exogenous damage caused by either natural environmental causes (ultraviolet [UV]-radiation, ionizing radiation, reactive oxygen species [ROS]) or synthetic genotoxic chemicals such as those present in tobacco smoke. Depending on the chemical nature of the damage on DNA, different types of repair programmes are evoked. UV radiation causes the formation of bulky DNA adducts, which stall replication and transcription machineries. This activates nucleotide excision repair (NER), a process involving a complex machinery of enzymes in damage recognition and repair ([1,2](#)). Damage causing by double-strand breaks (DSB), typically ionizing radiation (IR), is repaired by homologous recombination (HR) and by nonhomologous end-joining (NHEJ) ([3,4](#)).

DNA lesions are mainly recognized by damage-type specific sensor molecules. For example, the primary sensor activated by IR is ataxia teleangiectasia mutated protein (ATM), which phosphorylates several key checkpoint proteins and initiates a protein kinase cascade leading to the phosphorylation of a large number of its downstream targets. UV damage and stalled replication forks activate an analogous, but different

pathway involving kinases ataxia teleangiectasia and Rad3-related (ATR) and Chk1. In both cases, the respective protein kinase pathways dictate the subcellular responses and initiate damage repair (5–10).

Exposure of the cells to the various DNA lesions also causes other rapid cellular responses. On one hand, intracellular signaling cascades, such as JNK, p38, and growth factor receptor induced pathways are activated, and, on the other hand, cell-cycle checkpoints are enforced causing the arrest of the cells in specific phases of the cell cycle. While γ -radiation provokes both G_1 , intra-S, and G_2/M arrest, UV damage causes mainly a replicative arrest without distinct accumulation of the cells at any specific cell-cycle phase (11,12). Though the upstream activating events vary, the γ -radiation induced cell-cycle responses are largely mediated by a similar set of proteins controlling the normal cell cycle. The arrest of the cells in the cycle provides time for the damage repair, followed by activation of mechanisms mediated by polo-like kinase to reactivate the cycle (13). Thus, not only are the machineries ensuring adequate damage repair essential, but also the cell-cycle exit pathways are controlled.

Several of these features and molecules involved in the processes are currently being exploited for diagnostics and new strategic therapies, which involve attempts to both recover the repair deficiencies and to enhance tumor cell killing by provoking unscheduled cell death by preventing successful transcription-coupled repair. These new treatment modalities may offer effective therapeutic opportunities even in cases of severe mutational loads, such as in hereditary cancer repair syndromes. This chapter discusses the pathways involved in sensing the DNA damage and damage repair, the overlap between the cell cycle and DNA damage checkpoints, their defects in human syndromes, and how this knowledge is currently being exploited in therapeutic approaches.

2. Sensing the Damage

Cellular responses detecting DNA damage couple directly with DNA damage checkpoints and form a fast acting complex network with partially overlapping interactions. One of the key sensors is the ATM protein, which is a large phosphoinositide-3 kinase like kinase normally activated upon DSBs, and absent in AT patients because of its mutations (5,8,10). Activation of ATM leads to its dimerization and rapid autophosphorylation within minutes of the damage (14). ATM has a number of target proteins which function in the ensuing damage response and repair pathways (8,10). Interestingly, the fast activation kinetics of ATM and its absence from the DNA damage induced foci are indicative that ATM does not associate with the DNA lesions itself, but is rather activated by changes in chromatin conformation and orchestrates the subsequent events. ATM targets include proteins responsible for G_1 (p53, Mdm2, Chk2), intra-S (Nbs1, FancD2, SMC1), as well as G_2/M checkpoints (Brca1, Rad17) (see Chapter 4). The phosphorylation of histone γ -H2AX (H2AX) by ATM (15) leads to H2AX accumulation at the sites of DSB and formation of nuclear foci. Similarly, H2AX is phosphorylated by ATR in response to replicational stress (16). Whereas the lack of H2AX does not affect the DNA damage checkpoints per se, H2AX is facilitating the accrual of Nijmegen breakage syndrome 1 (Nbs1), p53 binding protein 1 (53BP1), and breast cancer-1 (Brca1) to the sites of the damage (17–19). Hierarchially, the recruitment of 53BP1, originally identified as a p53 interacting protein (20), to these foci (21,22), is essential for the subsequent accumulation and phosphorylation of other ATM targets Nbs1, cohesin protein structural maintenance of chromosomes

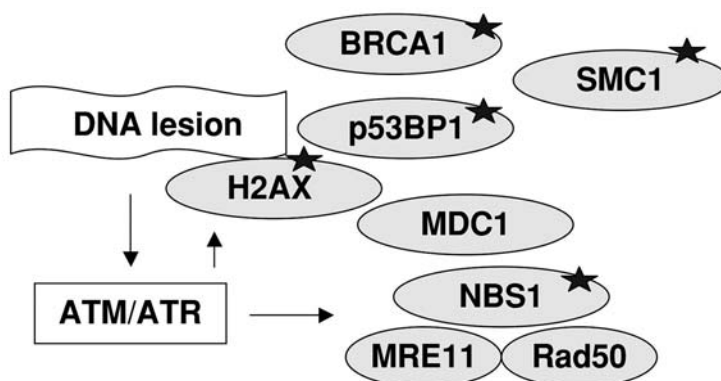


Fig. 1. DNA lesion induced recruitment of DNA damage response proteins depend on ATM/ATR initiated phosphorylation cascades. Proteins marked with star are targeted by ATM/ATR.

protein 1 (SMC1) and Brca1 and, at least partly, for phosphorylation of Chk2 (23–28; for review, *see ref. 29*) (Fig. 1). However, whereas H2AX is not required for the initial 53BP1 foci formation, it is essential for the maintenance of 53BP1 at or in the vicinity of the damage (21). Support for models placing ATM both upstream and downstream of 53BP1 exist (22). Foci containing H2AX, 53BP1, Nbs1, and mediator of DNA damage checkpoint 1/NFBD1 (Mdc1) are also detected in senescent cells and apparently represent activation of the DNA damage responses by increased number of telomere breakage (30). Mdc1 may further determine the sustained interactions of Nbs1 with DSBs and appears therefore essential for the dynamic interactions at the site of the damage (31).

A close homolog of ATM, ATR is activated by DNA helix distorting events, such as UV damage and DNA topoisomerase inhibitors, and shares an apparently similar substrate spectrum as ATM (5,10). However, ATR, but not ATM, is essential during embryonal development indicating that they also have diverse functions (32,33). ATR couples constitutively with its regulatory partner ATRIP (34) and the complex accumulates to the arrested replication forks (35). The ATR-dependent checkpoint activation is dependent on replication protein A (RPA)-mediated loading of ATR-ATRIP, Rad9-Rad1-Hus1 complex, Claspin and DNA polymerase α to the site of stalled replication causing the activation of the ATR dependent checkpoint (10,36).

3. Repairing the Damage

3.1. Repair of DNA Doublestrand Breaks

DSBs occur through exogenous DNA damage, typically by ionizing radiation or chemicals, or by replication of single-strand breaks (SSBs). Unrepaired DSBs sensitize the cells to chromosomal aberrations, aneuploidy, and deletions. DSBs are repaired by both HR and NHEJ. Whereas HR is essentially error free, NHEJ allows loss or gain of few nucleotides (2–4). In HR, an intact template of the sister chromatid is used to repair the lesion, whereas NHEJ operates by joining the DNA ends without sequence homology with the aid of Ku70/80 complex, DNA-PK catalytic subunit and XRCC4 ligase 4 (2,3). HR appears significant for the re-establishment of stalled replication forks due to DSBs, therefore allowing cell survival. Although NHEJ is constitutive, HR is more cell cycle-restricted

and dependent on exogenous stress signaling (37). HR is activated by the ATM/ATR pathway and requires a large number of molecules including Rad51 and its paralogs, Rad52, Rad54, Brca1, Brca2, XRCC2, XRCC3, and the MRE11, Rad50, Nbs1 (MNR) complex (2,3). The phosphorylation of Rad51 by Chk1 and their interaction is essential for successful HR again establishing a connection between checkpoint control and repair (38).

3.2. Repair of UV-Induced DNA Lesions

Nucleotide excision repair (NER) is activated in mammalian cells by bulky DNA helix-distorting lesions, such as pyrimidine dimers and 6-4 photoproducts caused by UV damage, or other adducts caused by certain chemicals and oxidative damage (39). NER is accomplished by the coordinated action of 20 to 30 proteins each with specific functions in damage recognition, unwinding of the DNA helix, excision of the damage site, ligation, and rewinding of the DNA helix (2,39). Most proteins in the recognition and incision steps have been identified and named according to the seven complementation groups of *Xeroderma pigmentosum* (XP, XPA to XPG), a photosensitivity disorder resulting from deficient NER (2). Importantly, the TFIIH transcription factor is involved not only in general cellular transcription, but also in the local unwinding around the injury through its DNA helicase activity (2). NER is accomplished via two mechanically distinct processes—transcription-coupled repair (TCR) and global genomic repair (GGR) (40,41). TCR is activated through stalling of the RNA polymerase II as a result of the bulky DNA lesions (42), whereas GGR is launched by XPC-hHR23B protein complex recognizing the UV type of DNA damage (43). TCR occurs rapidly on the transcriptionally active DNA template strand, and may show selectivity towards faster repair of shorter genes (44). GGR is slower and responsible for the repair of both nontemplate strand and the nontranscribed areas. In the event of unsuccessful TCR, the cell is destined toward apoptosis, thus effectively removing cells with irreparable damage (40,41). However, the genomic fidelity is influenced more by GGR, because the failure of GGR fidelity leads to passing of the lesions to the daughter cells and thus accumulation of the genetic defects.

During TCR, when repair enzymes are recruited, they must remove the RNA polymerase II to access the lesion. This occurs either by ubiquitination and the subsequent degradation of RNA polymerase II (45), its phosphorylation and unstable association with DNA (46), or by some other, still unresolved mechanism. In addition, TCR requires Cockayne syndrome proteins CSA and CSB. Whereas these are unessential for GGR, their deficiency results in the UV radiation hypersensitivity disorder Cockayne syndrome (47). Furthermore, UV damage causes local inhibition of transcription around the damaged areas in the nucleus through deprivation of TFIIH, which is required for both NER and transcription initiation (48).

4. Coupling of DNA Damage and Cell-Cycle Checkpoints

DNA damage checkpoints have evolved to ensure the genetic fidelity. In essence they are positions within the cell cycle when the surveillance mechanisms are active and scan for the presence of damage. If damage is detected, the checkpoint is activated (49–51). The activation of mammalian checkpoints provoke distinct cellular responses, including cell-cycle arrest at various points in the cycle (G_1 , intra-S, G_2/M) and/or apoptosis. In

principle, the enforcement of these checkpoints is believed to yield time for the cell for efficient repair and to prevent accumulation of the genetic lesions by forcing the cells into apoptosis (52).

4.1. G_1 and G_1/S Transition Checkpoints—The ATM, Chk1/2, p53 Cascade

TP53 gene is the most frequently mutated gene in all human cancers. p53 protein is activated by different forms of cellular stress like hypoxia, nucleotide imbalance, replicative stress, pH-change, and perhaps most importantly, by DNA damage (53). Activation of p53 leads to cell-cycle arrest, rendering time for damage repair, or induction of apoptosis to prevent division of the damaged cells. Therefore its key function is considered as a sensor for the integrity of the genome (54). p53 is negatively regulated by three E3 ubiquitin ligases, murine double minute (MDM)2, Pirh2, and COP1 initiating p53 proteasomal degradation (55–57). Of these, Mdm2 and COP1 are direct p53 transcriptional targets and form important regulatory loops controlling p53 function. p53 and MDM2 are directly phosphorylated by ATM following IR and p53 by ATR (58–60; reviewed in ref. 61). Chk2 further phosphorylates p53 on its aminotermius (62,63). The phosphorylation of p53 to multiple sites may negatively regulate its association with MDM2 and affect the subsequent p53 transactivation and its interaction with other modulators like p300 histone acetyltransferase (61,64). Though the lack of Chk2 does not affect p53 stabilization following damage, Chk2 null cells and animals display a defect in p53-dependent transcription and apoptotic response to IR (65).

p53 exerts its functions by acting as a transcription factor regulating multiple target genes involved in cell-cycle regulation and apoptosis, but also in cellular signaling and regulation of extracellular matrix and cellular structures (66). The key p53 target leading to a cell-cycle arrest is p21Cip1/Waf1, which inhibits the activities of cyclin-dependent kinase (Cdk)2-cyclin complexes required for the S-phase entry of the cells (67,68). The major function of p21 is to provide a sustained downregulation of the Cdk2/cyclin E complex activity both in G_1/S transition and in S-phase (Fig. 2).

p53 apoptotic targets include several mitochondrial proapoptotic genes, NOXA, PUMA, BAX, DR5 (reviewed in ref. 69). Moreover, p53 itself is directly localized to the mitochondrial inner membrane, binds with BclXL and Bcl2 (70) and releases proapoptotic proteins Bak and Bax (71,72). Therefore, in mammals the activation of the p53 function may lead to multiple cellular responses that affect the decision between cell-cycle arrest and apoptosis and have therefore therapeutic implications (73–75).

In *Drosophila*, the cellular DNA damage responses are dependent on the developmental stage, and presented with either a delayed mitosis or a premitotic block. The ATM provoked signaling cascade is fully conserved in *Drosophila*, and signaling through *Mei-41* (ATM/ATR), *Mnk* (Chk2), and *Dmp53* (p53) is required for IR induced apoptosis (76). Furthermore, the evaluation of DNA damage responses in *Drosophila* has been very informative of the functions of *Dmp53* in apoptosis. In response to γ -radiation, *Dmp53* is required for the apoptotic response, but not G_2 arrest. *Dmp53* transcriptional targets, induced by γ -radiation, include proapoptotic genes *reaper*, *sickle*, *hid*, and *Eiger*, but classical p53 cell cycle targets, like *Dacapo* (p21/p27) are missing (76). Furthermore, the *Drosophila* apoptotic response is dependent on *mnk*, the Chk2 homolog, phosphorylating p53. Therefore, the *Drosophila* fully recapitulates the upstream p53

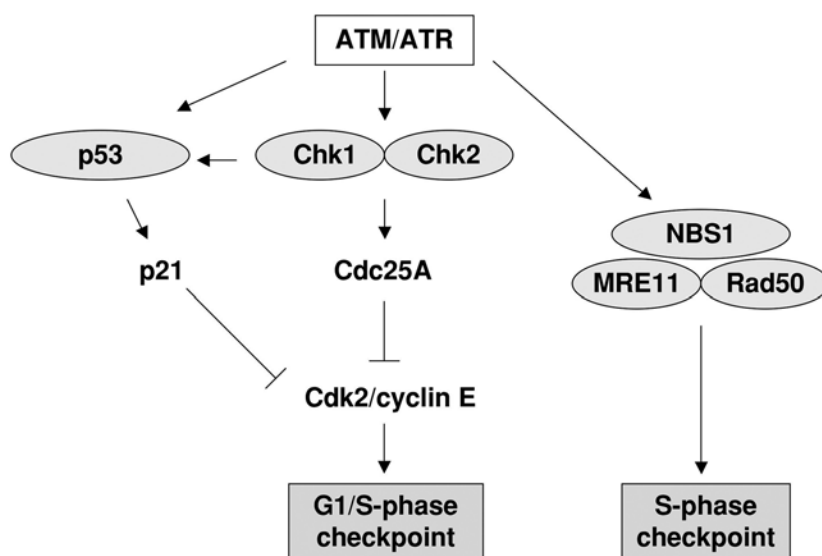


Fig. 2. G_1/S transition and intra-S phase checkpoints are enforced by parallel pathways.

DNA damage activation pathway and provides a model for further studies in damage checkpoint functions.

Progression of the mammalian cell cycle requires the activity of Cdc25 family of phosphatases, which remove the inhibitory phosphorylations of Cdks and thus enable Cdk-activities required for both S-phase (Cdk2) and mitosis (Cdk1) together with their cognate cyclins (77). During normal cell-cycle Cdc25A is phosphorylated by Chk1 and targeted for degradation, thus controlling the entry of the cells into S-phase (78,79). In response to both IR and replication blocks, Cdc25A is phosphorylated and degraded by the proteasome (80–82). IR-induced degradation of Cdc25A is provoked by ATM-Chk2 kinases, whereas the UVC-induced degradation occurs through the ATR-Chk1 kinase cascade (80,83,84). In consequence, the replicative cyclin E-Cdk2 complex is inactivated, S-phase arrest ensues (80) and prevents the loading of Cdc45 to replication origins (85) (Fig. 2). Both Chk2 and Chk1 are required for Cdc25A degradation in response to IR (84,86), whereas the activity of Chk1 is sufficient following UV or stalled replication forks (80). On the other hand, activation of both Chk1 and Chk2 occurs in senescent cells as a result of the presence of uncapped telomeres provoking a DNA damage response (30). These findings therefore couple cell-cycle control and damage control.

Interestingly, embryonic stem cells do not display a proper G_1 arrest following IR apparently because of centrosomal sequestration of Chk2, causing increased cell apoptosis (87). Also Chk1 is observed on centrosomes and controls cdk1–cyclin B complex activity (88). Chk2 targets also transcription factors E2F and promyelocytic leukemia protein PML, which through their activities may further modulate the cellular responses by promoting, for example, apoptosis (89,90). Indeed, by phosphorylating and activating E2F1 following DNA damage both Chk1 and Chk2 upregulate p73, a close family member of p53 (91).

4.2. Intra-S-Phase Checkpoints

During S-phase, the cell monitors the progression of DNA replication. Stalled replication forks, either caused by damage to DNA or depletion of ribonucleotides, generate a damage response and arrest of the cells before entry to G₂. UV-damage causes a reduction at the rate of DNA synthesis manifested by a decrease in chain elongation and replication initiation (11). However, in the presence of DSB, replication is not completely halted, but the S-phase progression caused by inhibition of origin firing is delayed (92). Thus, mechanistically, IR and, e.g., UV radiation impose distinct cellular stresses and damage responses.

Several ATM initiated pathways or targets are activated during S-phase following DNA damage either to ensure an intra-S-phase delay or to initiate repair processes. These include the Chk1 and Chk2 initiated phosphorylation cascades leading to degradation of Cdc25A (83,84) and a S-phase delay by maintaining the Wee1 mediated inhibitory Thr14/Tyr15 phosphorylation on Cdk2 thus inhibiting the Cdk2/cyclin E kinase activities (79) (Fig. 2). In consequence, the maintenance of the retinoblastoma protein in an E2F suppressive mode ensures further that E2F targets, several of which are required for the orderly progression through S-phase, are not upregulated. This is however contrasted by the findings that DNA damage also activates E2F through Chk2 mediated phosphorylation and promotes apoptosis (90,93). During normal cell cycle, the selection between apoptotic and cell cycle-promoting activities can be mediated by TopBP1 which, by recruiting Brg1/Brm, a SWI/SNF chromatin-remodeling complex factor, to E2F1-responsive promoters, represses E2F1 activity (94).

The MRN complex, composed of Mre11, RAD50, and Nbs1 subunits, ensures the fidelity of the replication complex (95). The trimeric MRN complex is formed upon Nbs1 phosphorylation in an ATM/H2AX-dependent manner. Another ATM substrate, Mdc1 is localized to DSB foci and functions to mediate the interactions of H2AX and the MRN complex (96–98) (Fig. 2). Nbs1 is rapidly recruited to the site of the damage, whereas Chk2, though contacting the DSBs, appears to be a mobile transmitter of the damage signaling cascade (99). The MRN complex enforcing an intra-S-phase dependent checkpoint acts thus in parallel with Chk2-Cdc25A-Cdk2 to mediate S-phase arrest (85) (Fig. 2).

The capacity of cells to pass unrestricted through S-phase in the presence of DNA damage is called radioresistant DNA synthesis (RDS). This is a property shared by ATM-defective cells and cells null or carrying mutations of 53BP1, Brca1, Mre11, and FancD2 (22), whereas Nbs1 and ATLD cells show partial RDS (85). Interestingly, radioresistant DNA synthesis couples often with radiosensitivity of the cells apparently reflecting their increased genomic instability. Thus ATM, Chk1, 53BP1, H2AX, Mdc1, Nbs1, SMC1, Brca1, and FancD2 defective cells display increased radiosensitivity (10,22,79,100). FancD2, mutated in Fanconi anemia (FA), a chromosomal instability disorder with high frequency of cancers, is also a target of ATM (101), and interacts with Nbs1 (102). Thus, AT, NBS, and FA patients and 53BP1 null mice are cancer prone, suggesting that they have a critical role in damage provoked checkpoints (101,103). The increased radiosensitivity in the presence of a checkpoint defect could reflect unsuccessful repair, increased transcriptional stress and/or mitotic catastrophe. Yet, at the same time these features also predispose to increased genomic instability and malignant conversion.

ATR, on the other hand, appears to act as a surveillance mechanism of both DNA replication and for stalled replication forks. Similar to the ATR-ATRIP complex binding to ssDNA during replicative stress, ATR, Claspin, Rad9-Hus1 complex are required during the normal replicative cycle (36). In *Xenopus* extracts, DNA replication is a prerequisite for recruitment of ATR and Rad1 to chromatin and for damage-induced phosphorylation of Chk1 (104). Although the UV damage induced S-phase checkpoint response is mediated through ATR signaling through Chk1, it is independent of ATM, Nbs1, and Mre11 (105). Whether replicative stress causes further activation of ATR or changes its substrate spectrum remains to be determined.

4.3. Mitotic Checkpoint Control

The error-free segregation of the sister chromatids during mitosis is essential for the division of equal copy numbers to the daughter cells. Frequent aneuploidy in tumors is a characteristic feature of dysfunction of this critical checkpoint. In response to unreplicated DNA or DNA lesions, cells arrest before entry into mitosis and accumulate to G₂ (106). Later in mitosis, the spindle assembly checkpoint is triggered if unattached kinetochores are present and to inhibit the anaphase entry. Kinases (Chk1, Chk2) and phosphatases (Wee1) acting on the absolutely essential mitotic kinase, Cdk1, are the core machinery of this checkpoint (106). Following DSB or replication stress, Chk1/Chk2 phosphorylate dual specificity phosphatases Cdc25C and Cdc25A, and provoke their subsequent proteasomal degradation (80; reviewed in ref. 107). This leads to inactivation of the mitotic kinase Cdk1 by maintaining its inactivating Thr14/Tyr15 phosphorylations and G₂/M-phase arrest (86). Furthermore, 14-3-3 proteins associate with and may mediate the relocation of both Cdc25B and Cdc25C to the cytoplasmic compartment (107,108). p53 may contribute to this event by inducing the expression of 14-3-3sigma (109). Conversely, during mitosis, cyclin B-Cdk1 phosphorylates Cdc25A, leading to its stabilization and allowing its mitotic activity (110). Polo-like kinase, a mitotic kinase which activates Cdk1-cyclin B complex, is inhibited following DNA damage (111) further ensuring the mitotic arrest.

BRCA1 deficiency leads to gross chromosomal changes and mitotic abnormalities, which correlate with a deficiency in homologous recombination and DSB repair (100). Both Brca1 and Brca2 are expressed during S- and G₂-phases of the cell cycle. Following DSB, Brca1 translocates to the lesions and is phosphorylated by ATM, ATR, and Chk2 (17,23,60). Furthermore, it colocalizes with FancD2 and Rad51 and interacts with the MRN complex (100). The multiple Brca1 interactions may be organized through recognition and binding of phosphoprotein domains via its BRCT-domain (112,113), present also in 53BP1 and Mdc1. Interestingly, Brca1 is required for the activation of Chk1, down-regulation of Cdc25C and the mitotic checkpoint (114), implicating extensive crosstalk in the damage response pathways. Brca2 can also be found in the DNA damage foci containing Brca1, FancD2, and Rad51, which occurs through FancD2 mediated monoubiquitination of Brca2 (115). Brca2 and FancD2 null cells show chromosomal instability indicating that they participate in transcriptional repair (115).

The spindle assembly checkpoint is governed by proteins that sense and bind unattached kinetochores such as the Mad and Bub proteins (116). Their function is required for the mitotic checkpoint in both yeast and mammalian cells. Bub1 is mutated in a subset of colorectal cancers (117) suggesting that its dysfunction in mitotic control

predisposes to tumorigenesis. Furthermore, 53BP1 localizes to the kinetochores of mitotic cells suggesting that it may function also in the mitotic checkpoint. Indeed, cells deficient for 53BP1 manifest a G₂/M checkpoint defect following low, but not high doses of IR (25,26). This suggests that high doses of DNA damage invoke additional surveillance mechanisms independent of 53BP1.

5. Hereditary Syndromes With Increased Predisposition to Cancer Couple With Defects in Damage Sensing and Repair

Several rare autosomal recessive disorders are characterized by spontaneous chromosome breakage. AT patients have an increased risk of cancers, but also progressive cerebellar ataxia, immune deficiencies, and other malformations (5,8) (Table 1). Nijmegen breakage syndrome shares several clinical features with AT, but the NBS patients have also microcephaly and distinguished facial features (118). AT-like disorder (ATLD) patients with mutations in the *MRE11* subunit of the MRN complex, resemble those of AT patients and have also chromosome instability (Table 1). Whereas AT, ATLD, and NBS have partially overlapping phenotypic features, a mutation of *ATR* in Seckel syndrome produces mostly congenital abnormalities with severe growth and mental retardation and microcephaly. Fanconi anemia (FA) is a multigenic syndrome with chromosome instability and overlapping clinical features including skeletal abnormalities and bone marrow failure (119). Hereditary breast cancer associated genes, *BRCA1*, *BRCA2/FANCD2*, and *CHK2* are intimately coupled with the damage signaling networks through their direct associations and by coupling with the recombination and repair machineries. Lastly, Li-Fraumeni syndrome patients, with multiple types of early-onset cancers, have germline mutations of *TP53* and *CHK2* (120). It is striking that of the genes involved in the DNA damage response and damage checkpoint pathways, only *MDC1*, *SMC1*, *53BP1*, and *H2AX* are yet to be coupled to either human cancer syndromes or other developmental abnormalities.

Mutations of DNA repair-associated genes in hereditary syndromes have been reviewed recently and are not discussed here (2,115).

6. New Therapeutic Approaches Through Interference of the DNA Damage Response Pathways

The sensitivity of the damage checkpoint pathways have presented the possibility that revoking their activities connecting with the apoptosis pathways could be utilized in strategies for new treatments. One such example is β -lapachone, an S-phase checkpoint activator and topoisomerase I inhibitor, which activates E2F1 and selectively kills tumor cells by inducing apoptosis (121). Attempts have also been based on specific abrogation of the damage sensory processes or damage checkpoints. There are numerous examples of radiosensitivity in cells with defective damage responses and repair mechanisms. Therefore, several approaches have been undertaken to search for compounds abrogating the proper checkpoints thus causing chemosensitivity. For example, UCN-01, which selectively inhibits Chk1, is being tested as a chemosensitizing agent in clinical phase I/II trials in combination with several topoisomerase inhibitors and antimetabolites (122). Several lines of experimental evidence have provided support that downregulation or inhibition of the pathway mediators, like silencing of ATM, ATR, or DNA-PKcs by RNAi based methods (123), provides enhanced killing of the

Table 1
Hereditary Cancer Syndromes With Mutations in DNA Damage Sensory and Checkpoint Proteins

Syndrome	Gene	Locus	Protein	Function	Clinical syndrome
Ataxia telangiectasia	<i>ATM</i>	11q22	ATM	Damage sensor	Cerebellar ataxia, teleangiectasia immune defects, lymphoma, leukemia
ATR-Seckel	<i>ATR</i>	3q22-24	ATR	Damage sensor	Growth retardation, microcephaly, mental retardation
AT-like disorder (ATLD)	<i>MRE11</i>	11q21	Mre11	Damage signaling	Cerebellar ataxia, chromosomal instability
Nijmegen breakage	<i>NBS1</i>	8q21	Nbs1	Damage signaling	Growth retardation, immunodeficiency, microcephaly, chromosomal instability, lymphoma
Fanconi anemia*	<i>FANC</i>	3p25.3	FancD2	Checkpoint control and homologous recombination	Congenital abnormalities and malformations, bone-marrow failure, leukaemia, skin changes
Hereditary breast cancer	<i>BRCA1</i> <i>CHK2</i> <i>BRCA2</i> (<i>FANCD1</i>)	17q21 22q12 13q12-13	Brcal Chk2 Brca2	Signal processor/ repair Damage signaling Repair	Breast and ovarian cancer
Li-Fraumeni	<i>TP53</i> <i>CHK2</i>	17p13 22q12	p53 Chk2	Transcription factor Damage signaling	Breast carcinoma, soft tissue sarcoma, brain tumors, osteosarcoma, leukemia

*Major known mutated genes are shown.

tumor cells. However, examples also exist of the presence of multiple pathway defects in tumor cells, affecting G₁, S, G₂/M checkpoints (p53 and Brca1 mutations) which do not sensitize the cells to enhanced death (124).

Miscuing of the cell-cycle checkpoints can also enhance cell killing. For example, the premature activation of Cdk1 has been shown to lead to apoptosis (125) and inhibition of Wee1 activity by PD166285 radiosensitizes p53 mutant cells lacking a functional G₁ checkpoint (126). The PD (pyrido [2,3-d] pyrimidine) compounds were initially synthesized as ATP competitive tyrosine kinase inhibitors and found to possess activities against Src, PDGF receptor and Abl-family tyrosine kinases (127). These compounds inhibit either S-phase (PD179483, PD166326) or mitosis (PD173955) (128). The S-phase inhibitors act in vitro as Wee1 inhibitors, have demonstrated activity in murine models of chronic myeloid leukemia (129) and could therefore be useful in combination therapies.

Several transcriptional inhibitors may in fact promote cell death by provoking transcriptional stress and thereby activation of the ATR pathway. Cdk-inhibitors flavopiridol and roscovitine block the catalytic activity of Cdks and also inhibit RNA polymerase II C-terminal phosphorylation thereby causing global inhibition of transcription. Therefore they not only arrest cell-cycle progression but also inflict transcriptional stress (130,131). Several cytotoxic drugs currently in clinical use act by causing DNA damage and by inhibiting transcription (alkylating agents, antimetabolites, topoisomerase inhibitors) (122,131). However, these have severe side effects, which calls for the need of more specific drugs or combination therapies.

There is substantial evidence that *TP53* mutations associate with increased radioresistance and chemoresistance (73–75). On the other hand, if p53 function is excessively activated during therapy, it can inappropriately promote death of radiosensitive normal cells. Therefore the p53 pathway has been an attractive target either in attempts to activate the mutant forms of p53 or to prevent the activity wt p53 (132). Examples exist for compounds activating wild type (Nutlins) (133) and mutant forms of p53 (Prima) (134) or inhibiting wild type p53 (Pifithrin) (135).

Lastly, because several hereditary repair syndromes have specific defects in one or more of the repair proteins, their replacement or increased repair by other means could provide a basis for effective therapy. In mouse models, the expression of photolyase, a cyclobutane pyrimidine dimer correcting enzyme absent in mammals, reduces skin damage (erythema, hyperplasia, apoptosis) (136,137). On the other hand, topical administration of pTT thymidine dimers mimic the UV-induced pyrimidine dimers and provoke an extensive damage response with upregulation of p53 and apoptosis (138). This treatment effectively enhances the rate of repair and diminishes the amount of UV induced skin cancer in mouse (139).

7. Conclusion

A substantial amount of information has been acquired on the players and pathways reacting to DNA damage. Detailed information has been accumulated on how dysfunction of the various components affect the cellular damage responses and predisposition to cancer. New strategies for treatment have been and are being designed to utilize this knowledge in various ways, including both damage reparative measures and measures imposing excessive damage or stress to provoke cell death. It is anticipated that these

approaches will provide more targeted and effective treatments with less drug-related adverse reactions.

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c-Myc, Apoptosis, and Disordered Tissue Growth

Michael Khan and Stella Pelengaris

Summary

Deregulated expression of *c-Myc* is present in most, if not all, human cancers and is associated with a poor prognosis. The *c-Myc* proto-oncogene is essential for both cellular growth and proliferation, but paradoxically may also promote cell death. The study of this “dual potential” of *c-Myc* over the past two decades has provided a paradigm for exploring the role of other mitogenic proteins, such as E2F, many of which have now also been shown to have such intrinsic “tumor suppressor” properties. In fact, it may be a general feature of proteins that promote cell cycle that the oncogenic potential of deregulated expression is restrained by concurrent activation of processes, such as apoptosis or senescence, which effectively prevent propagation of the “damaged” cell. By implication, these in-built “failsafe” mechanisms must be overcome during tumorigenesis. However, once prevented, for instance by inactivation of the p53 or Rb pathways or upregulation of antiapoptotic proteins, the potentially devastating oncogenic potential of proteins such as *c-Myc* is unmasked.

It is also likely that highly conserved proteins such as *c-Myc*, situated upstream of signaling pathways regulating both cellular replication/growth on the one hand and apoptosis/growth arrest, act as key integrators of processes determining cell numbers and tissue size during normal development and adult tissue homeostasis. Therefore, maybe not surprisingly, *c-Myc* may also contribute to disease by way of stimulating apoptotic pathways, the very process that also restrains their oncogenic potential; an expanding body of recent evidence implicates *c-Myc* as a contributor to the death of insulin-secreting β -cells in diabetes.

In trying to unravel the diverse roles of these enigmatic proteins and to explore their potential as therapeutic targets, the application of regulatable transgenic mouse models has proved invaluable. Such models have led us to reassess prevailing views of oncogenesis—particularly as they suggest that cancer formation may occur on a “minimal platform” of as few as two strategically placed oncogenic lesions. This has become the source of much debate, particularly following elegant studies showing that transformation of human cells in vitro requires a greater number of genetic lesions than equivalent rodent cells, a view that would seem to challenge the “minimal platform” model. It now seems likely that a greater number of individual signaling pathways require targeting for oncogenesis in man (including p53, Rb, telomere length, and others—at least in cultured cells) compared with mouse, though this does not seem to be the case if *c-Myc* expression is deregulated. So how do we reconcile this? *c-Myc* appears to be a special case (though there are likely others) as deregulation clearly interferes with multiple cancer-relevant signaling pathways and thereby induce multiple “hallmark” features of cancer. For this reason, *c-Myc* is also a potentially key therapeutic target, a view that is encouraged by the regression of even advanced and genetically unstable tumors, seen following deactivation of *c-Myc* in various models. Increasingly, and based on the logical assumption that the range of *c-Myc* induced phenotypes are driven primarily by the transcriptional activation of various target genes, many researchers are exploiting post-genome era technologies, for global gene expression and proteomic analyses, in order to define the activities of *c-Myc* in physiology and disease at a molecular level.

However, before we get too carried away, it must be emphasized that conditional mouse cancer models are increasingly reproducing the more unfortunate aspects of their human counterparts;

including the development of “escapers,” cancer cells that have become independent of the initiating mutations and thereby fail to regress, and the presence of so-called dormant cancer cells, which remain in apparently fully regressed tumors and eventually contribute to tumor recurrence. There is still a long way to go. This chapter will discuss these issues with a particular focus on *c-Myc*.

Key Words: *c-Myc*; apoptosis; growth; cell cycle; differentiation; cancer; diabetes; regression; gene expression.

1. Introduction

The *c-Myc* proto-oncogene encodes a transcription factor, c-Myc, the cellular homolog to the viral oncogene (v-*Myc*) of the avian myelocytomatosis retrovirus (1). It was shown some time ago that activated oncogenic *c-Myc* is instrumental in the progression of a human cancer, Burkitt’s lymphoma (2). More recently, elevated or deregulated expression of c-Myc has been detected in a wide range of human cancers, and is often associated with aggressive, poorly differentiated tumors (3–6).

The normal key biological function of c-Myc is to promote cell-cycle progression. However, as will be discussed, this enigmatic protein appears to be a key player in various other biological processes, such as differentiation, cell death, and angiogenesis. c-Myc is a transcription factor that requires dimerization with another protein, Max, in order to become transcriptionally active. Thus, most of its effects on cell behavior are likely to be the result of expression (and in some cases repression) of various target genes.

In the intact organism, c-Myc is expressed ubiquitously during development in growing tissues (7,8). Likewise, in the adult body, c-Myc is expressed in tissue compartments possessing high proliferative capacity (e.g., skin epidermis and gut), whereas it is undetected in cells that have exited the cell cycle. There also exist other homologs of c-Myc (i.e., L-Myc and N-Myc), and the expression of each is normally restricted to certain tissues. Aside from c-Myc expression correlating well with proliferating tissues in vivo, this protein is crucial for normal development, as deletion of both c-Myc alleles in the mouse leads to embryonic lethality (9).

With respect to human cancer, oncogenic *c-Myc* is instrumental in the progression of Burkitt’s lymphoma resulting from a translocational event. In addition, elevated or deregulated expression of c-Myc has been detected in numerous human cancers, such as breast, colon, cervical, small cell lung carcinomas, osteosarcomas, glioblastomas, melanoma, and myeloid leukaemias. In most cases the causes of c-Myc overexpression are not known, but include gene amplification, whereby multiple copies of the *c-Myc* gene arise under the influence of genome instability.

Not surprisingly, given the well described dangers of deregulated c-Myc expression there are a myriad of cellular processes able to restrain c-Myc. Thus, transcription of the *c-Myc* gene is regulated by positive and negative growth signals and subsequently various factors influence the ability of c-Myc to regulate gene expression. The c-Myc protein is subject to phosphorylation, acetylation ubiquitinylation, and rapid degradation or may be expelled from the nucleus or even placed under “house arrest” in the nucleolus. Thus, many factors able to interfere with this system can also affect c-Myc levels, but their role in cancer remains to be fully described.

Not surprisingly, considerable efforts are being devoted to developing therapeutics to target aspects of c-Myc activity. To this end, proof of hypothesis and much important information has derived from regulatable mouse tumor models (discussed later)—these

models have exposed various oncogenic properties of c-Myc during tumor progression as well as determining whether inactivating c-Myc in tumors can lead to their regression. Not surprisingly, a major challenge is achieving specific delivery of the c-Myc inhibitor only to the nucleus of tumor cells *in vivo*, an issue that is relevant for many therapeutic agents that target specific tumorigenic proteins.

Even, where c-Myc itself may not prove a viable therapeutic target, some of those genes/proteins whose expression is regulated by c-Myc might do. Over the past few years a large number of studies have employed gene chip microarrays, serial analysis of gene expression (SAGE), proteomics, and other high-throughput techniques to identify potential c-Myc-targets in cultured cells *in vitro*. In general, as recently proposed following elegant studies from the laboratories of Sedivy and colleagues; c-Myc-activated genes are indicative of a physiological state geared toward the rapid utilization of carbon sources, the biosynthesis of precursors for macromolecular synthesis, and the accumulation of cellular mass. In contrast, the majority of c-Myc-repressed genes are involved in the interaction and communication of cells with their external environment, and several are known to possess antiproliferative or antimetastatic properties (10). Gene expression has been examined in c-Myc activated basal keratinocytes *in vivo*, but apoptosis is not a feature of this model (11); results are in line with predictions about growth regulatory properties of c-Myc. In fact, based on studies where c-Myc is activated in cells previously rendered c-Myc null, it has been estimated that as many as 10% of all genes may be regulated by c-Myc activation in some way (12,13). However, all these studies, despite their undoubted utility, do not give us any direct indication of which of the many genes potentially regulated by c-Myc are actually responsible for mediating the different and even opposing outcomes of c-Myc activation. That this must be undertaken by examining the full repertoire of c-Myc phenotypes *in vivo* is essential; it is clear that c-Myc-induced cellular phenotype is absolutely dependent on tissue location *in vivo* (14,15); as will be discussed later, even the decision of whether c-Myc activation will result in growth and replication or death of the cell depends on environmental cues and epigenetic context. Thus, at present, despite important recent advances in describing putative c-Myc target genes/proteins many key questions remain unanswered.

In order to identify new therapeutic targets in c-Myc-induced responses we must first answer the following key questions.

1. How does c-Myc exert these distinct cellular outcomes—namely proliferation, growth, loss of differentiation, and apoptosis? Are they dependent on the transcription or repression of specific distinct or overlapping sets of c-Myc target genes?
2. To what extent do cellular responses to c-Myc depend on cell type or, more importantly, on tissue location, which will dictate the signals a given cell type receives from cellular interactions between extracellular matrix and neighboring cells?
3. How might circulating or paracrine/autocrine signals, able to mitigate particular c-Myc functions, be regulated?

The recent genetic construction of mice in which the expression or activation of a given gene/protein can be switched on or off *in vivo*, has provided a means to assess the physiological roles of individual (or combinations of) genes and their protein products in tumorigenesis in the all-important context of the intact adult organism (reviewed in ref. 16). Thus, we may now address how host and tumor cells communicate in induced

tumors, as well as validate a given gene/protein as a therapeutic target by simply switching off expression of the oncogene and looking for phenotype reversal (such as tumor regression). Such studies will have important implications for designing future therapies targeted to cancer cells.

2. Regulation of c-Myc Activity

Transcription of the *c-myc* gene is increased markedly at the G_0 – G_1 transition, when resting cells are induced to proliferate, and is normally tightly controlled by external signals including growth factors, mitogens, and β -catenin, which promote and factors such as transforming growth factor β (TGF- β), which inhibit.

When a normal cell in vitro receives a signal to proliferate, such as that following binding of a growth factor to its transmembrane receptor, the result is activation of signaling pathways such as the Raf-mitogen activated protein kinase (MAPK) cascade inside the cell that will ultimately lead to changes in gene expression. Consequently, the induction of specific genes will activate the cell-cycle engine. The *c-Myc* proto-oncogene is one of these induced genes, known as an early-response gene (induced within 15 min of growth factor treatment in vitro) and plays a crucial role in allowing cells to exit G_0 and to make the transition from G_1 to S.

Various other factors may influence activation of c-Myc. β -catenin plays a signal-integrating role in Wnt- and growth factor-dependent proliferation events in mammalian development by both derepressing several classes of repressors and by activating Pitx2, which in turn regulates the activity of several growth control genes, including c-Myc (17). The activation of lymphoid enhancer factor (LEF)/T-cell factor (TCF)-mediated transcription by sustained expression of β -catenin and the loss of TGF- β signaling are essential steps in carcinogenesis, particularly for cancers of the colon, breast, and liver. Both of these signaling pathways can target c-Myc. LEF/TCF-responsive elements are found in the promoter of the human *c-Myc* gene and β -catenin activates the transcriptional activity of the c-Myc promoter by binding to this element in various cell lines. When TCF, but interestingly not LEF, is bound to this element, TGF- β dissociates β -catenin and represses the transcriptional activity of the *c-Myc* promoter (18), suggesting that under situations where LEF predominates the ability to downregulate c-Myc by TGF could be impaired. c-Myc is also a target gene of the c-Myb transcription factor, which is often over expressed in leukaemia and epithelial cancers (19).

In most cases the causes of c-Myc overexpression remain to be specifically described, but include gene amplification, whereby multiple copies of the *c-Myc* gene arise under the influence of genome instability, which accompanies advancing neoplasia. Recent data suggest that the stability of the c-Myc protein may itself be regulated. Cells have evolved a number of mechanisms to limit the activity and accumulation of c-Myc. One of the most striking of these mechanisms is through the ubiquitin-proteasome pathway, which typically destroys c-Myc within minutes of its synthesis, thus many factors able to interfere with this system can also affect c-Myc degradation (20–22) (see Fig. 1).

2.1. c-Myc Turnover

The turnover of c-Myc is at least in part dependent on phosphorylation of two highly conserved residues in MB1 that are mutated in v-Myc (the viral oncogenic Myc) and in various cancers; phosphorylation at Ser 62 stabilizes c-Myc, whereas subsequent

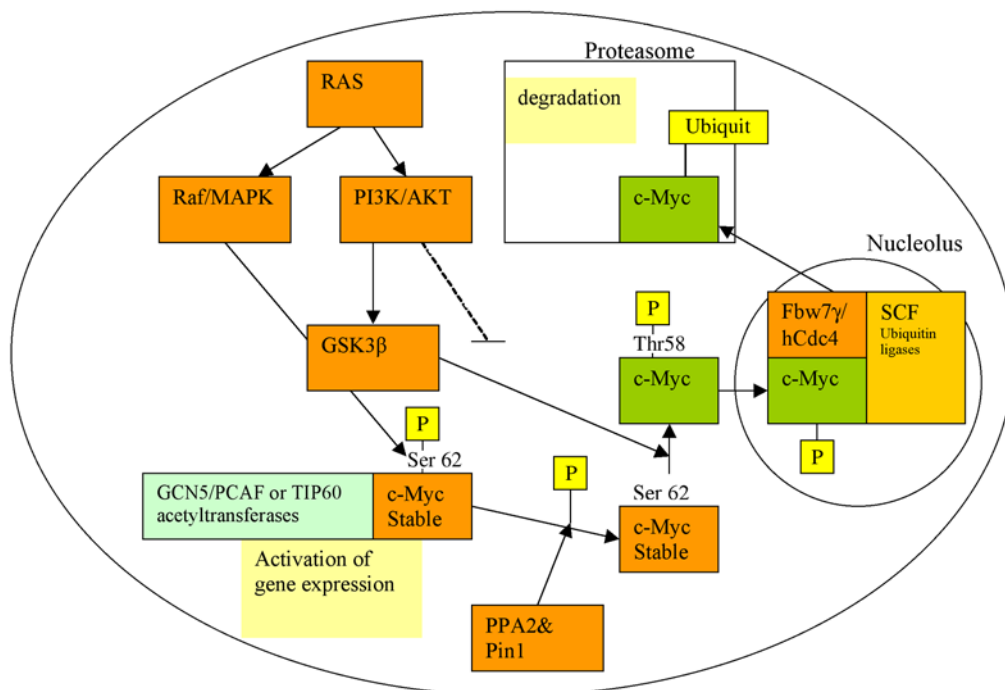


Fig. 1. Effects of acetylation and of Ras signalling on c-Myc protein stability. The MAPK pathway can stabilize c-Myc by phosphorylation of Ser62. Acetylation by acetyltransferases can also stabilize c-Myc. Conversely, c-Myc is destabilized by removal of the phosphate on Ser 62, by the phosphatases PPA2 and Pin1, and thence by phosphorylation of Thr 58 by the enzyme GSK3 β . Thr 58 phosphorylation targets c-Myc for ubiquitinylation by Fbw7 and SCF ligases. This ubiquitinylation may in part take place in the nucleolus. PI3K may contribute to stabilisation of c-Myc by preventing phosphorylation on Thr58.

phosphorylation at Thr 58 and dephosphorylation at Ser 62 are needed prior to ubiquitinylation of c-Myc and degradation in the proteasome. Several pathways have now been identified that play key roles in determining c-Myc phosphorylation, particularly those regulated by Ras. Thus, via the Raf–MAPK cascade, c-Myc can be phosphorylated on Ser 62, which stabilizes c-Myc, but may also be a necessary step prior to further phosphorylation at Thr 58 by glycogen synthase kinase 3 β (GSK-3 β). Removal of the phosphate at Ser 62 is mediated by the protein phosphatase PPA2 and the isomerase Pin1, two other Ras-regulated enzymes (23). Conversely, via the PI3K/Akt pathway, Ras can inhibit GSK-3 β , during early G₁, preventing phosphorylation of Thr 58 and degradation of c-Myc. However, the subsequent decline in Akt later in G₁, allows Thr 58 phosphorylation and c-Myc degradation.

The ability of c-Myc to activate transcription depends on the recruitment of several cofactor complexes including histone acetyltransferases (discussed later). In fact, acetylation of nucleosomal histones has long been recognized as a major regulator of gene transcription, but only recently has it been discovered that acetylation may also regulate subcellular localization and protein turnover. Thus, in a very recent study it has been demonstrated that degradation of c-Myc may also be regulated by acetylation by the acetyltransferases mGCN5/PCAF and TIP60, which enhance protein stability (24). As

these same enzymes, alongside others, seem important in mediating transcriptional activation by c-Myc this raises the intriguing possibility that at least in part this may involve stabilizing of protein levels.

The final step in c-Myc degradation is ubiquitinylation, which targets the protein to the proteasome for degradation. The human tumor suppressor Fbw7/hCdc4 acts in a complex with SCF ubiquitin ligases to catalyze the ubiquitinylation of c-Myc. One particular isoform the Fbw7 γ may regulate nucleolar c-Myc accumulation and ubiquitinylation by binding the c-Myc box1 (MB1) domain (22). Recent studies have indicated how complex the regulation of c-Myc may prove to be, as not all c-Myc is necessarily subjected to the same rates of turnover; a pool of c-Myc that is metabolically stable has been identified (25).

2.2. Transcriptional Activity of c-Myc

c-Myc activates a diverse group of genes as part of a heterodimeric complex with its partner protein, Max. The C-terminal basic helix-loop-helix leucine zipper (bHLH-LZ) domain of c-Myc binds Max, also a bHLH-LZ protein, to form c-Myc-Max heterodimers that are capable of binding specific DNA sequences, such as the E-box sequence CACGTG (26). The 150 amino acid N-terminal region of c-Myc contains two highly conserved elements, c-MycBoxI (MBI) and MBII, and is required for transactivation of target genes. Importantly, mutations in the c-Myc transactivation region, or in the bHLH-LZ domain required for dimerization with Max and DNA binding abolish c-Myc's effects on cell proliferation, transformation and apoptosis (27). c-Myc's functional domains as well as various c-Myc-interacting proteins involved in transcriptional regulation (28), are shown in Fig. 2. One way in which c-Myc-Max heterodimers can activate gene expression is by displacement of the putative tumor suppressor Mnt from target genes (29).

Exciting data provide further understanding of how c-Myc-Max complexes regulate gene activation—through chromatin remodeling (30–32). c-Myc was found to associate with the coactivator TRRAP, a component of a large complex that contains histone acetyltransferase (HAT) activity. Bouchard et al, (33) showed that upon binding the cyclin D2 promoter, c-Myc recruits TRRAP and induces the preferential acetylation of histone H4 at a single nucleosome. This would alter chromatin structure, thus allowing accessibility and binding of c-Myc-Max transcriptional activator complexes to target DNA sequences. Furthermore, c-MycBoxII in the N-terminal region of c-Myc is required for TRRAP recruitment, histone acetylation, and transcriptional activation at the cyclin D2 locus. Correlation of H4 acetylation with induction of gene expression has been extended to several other c-Myc target genes (34). However, work from the laboratory of Farnham and coworkers has shown that acetylated H3 and H4 may not be augmented by c-Myc, at least with respect to the *cad* and *tert* genes (35).

Very recent studies provide further information on how c-Myc may silence gene expression by interfering with the function of other transcriptional activators by the active recruitment of corepressor proteins (36). c-Myc can bind the corepressor Dnmt3a and influence DNA methyltransferase activity in vivo. In cells with reduced Dnmt3a levels, c-Myc-repressed genes such as *p21Cip1* are reactivated, whereas there is no apparent effect on expression of c-Myc-activated genes. Importantly, the authors noted that c-Myc, Dnmt3a and Miz-1 form a ternary complex that may be responsible for corepression of the *p21Cip1* promoter.

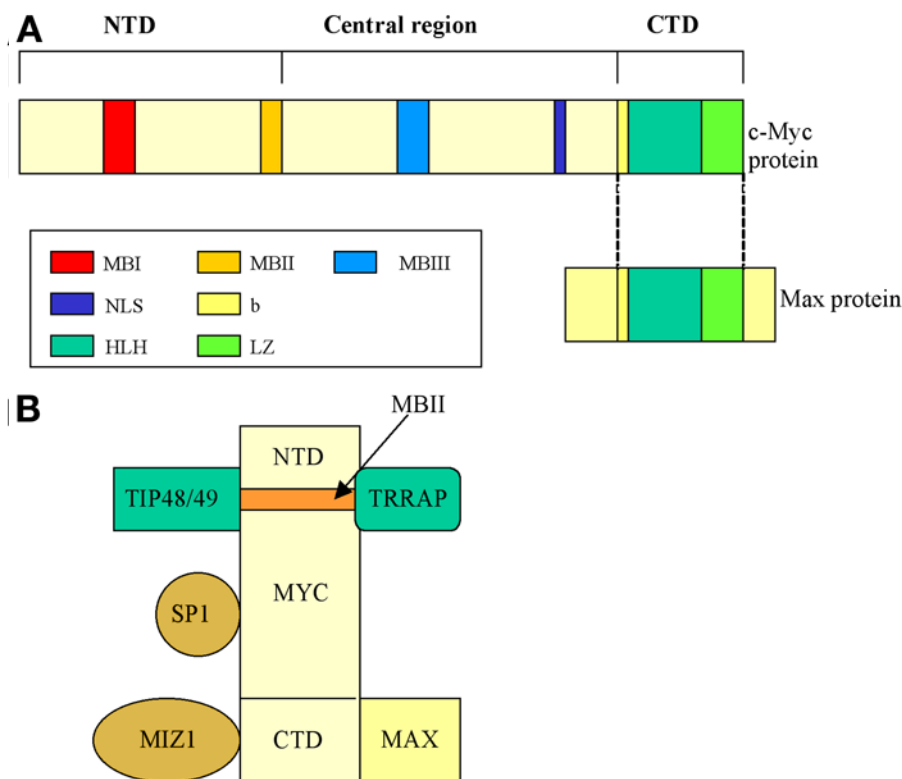


Fig. 2. Functional domains of human c-Myc protein. **(A)** The C-terminal domain (CTD) of human c-Myc protein harbors the basic (b) helix-loop-helix leucine zipper (bHLH-LZ) motif for dimerization with its partner, Max, and subsequent DNA binding of c-Myc-Max heterodimers. The N-terminal domain (NTD) harbours conserved “c-Myc boxes” I and II (MBI and MBII), essential for transactivation of *c-Myc*-target genes. Recently the MBIII situated in the central region has been found to be important for negatively regulating the apoptotic response. **(B)** Some major c-Myc-interacting proteins that may or may not bind simultaneously to c-Myc. These include coactivator TRRAP, part of a complex possessing histone acetylase activity (HAT), which interacts with MBII region and mediates chromatin remodelling. TIP48 and TIP49 proteins interact with the NTD of c-Myc and are implicated in chromatin remodelling as a result of their ATP-hydrolyzing and helicase activities. Proteins involved in transcriptional regulation, such as Miz-1 interact with the CTD of c-Myc, whereas SP1 interacts with the central region of c-Myc. NLS; nuclear localization signal.

Recent studies have begun to reveal an important role for the highly conserved c-Myc box III (MbIII) region, situated in the central region of the protein. MbIII is important for transcriptional repression by c-Myc, and for transformation *in vitro* and lymphoma development *in vivo*. Conversely, disruption of MbIII prevents transformation by increasing the apoptotic activity of c-Myc, suggesting this region may be an important negative regulator of apoptosis activity of c-Myc (37).

2.3. c-Myc; Cell Growth and Proliferation

One of the key biological functions of c-Myc is its ability to promote cell-cycle progression (3,38–40). In quiescent cells *in vitro*, c-Myc expression is virtually undetectable. However, upon mitogenic or serum stimulation, c-Myc mRNA and

protein are rapidly induced and cells enter the G₁ phase of the cell cycle. Thereafter, *c-Myc* mRNA and protein decline to low but detectable steady-state levels in proliferating cells. If serum or growth factors are removed, *c-Myc* levels decline to undetectable levels and cells arrest (41). During development, enhanced *c-Myc* expression correlates well with active proliferation and its down-regulation accompanies mitotic arrest and onset of differentiation; targeted gene disruption of both *c-Myc* alleles in embryonic stem cells, leads to embryonic lethality at days 9.5 to 10.5 (7,8).

More recently, the cell-cycle effects of ablating *c-Myc* have been investigated. A rat fibroblast cell line in which both alleles of *c-Myc* were ablated shows greatly reduced rates of cell proliferation (42,43). A further prominent effect was the marked deficiency in cell growth, defined by decreased global mRNA and protein synthesis. Cell-cycle elongation is caused by a major lengthening of G₁ phase (four- to fivefold) and associated with significantly delayed phosphorylation of the retinoblastoma protein (Rb), and a more limited lengthening of G₂ phase (twofold), whereas S-phase duration is largely unaffected. Progression from mitosis to the G₁ restriction point and the subsequent progression from the restriction point into S phase are both drastically delayed, suggesting that *c-Myc* directly affects cell growth (accumulation of mass) and cell proliferation (the cell-cycle machinery) by independent pathways.

The first notion that *c-Myc* influenced cell growth came from the correlation between *c-Myc* and the expression of the rate-limiting translation initiation factors eIF4E and eIF2 α (44) now known to be direct *c-Myc* targets (45,46). More recently, *c-Myc* has been shown to have a direct role in the growth of invertebrate (47) and mammalian cells (48–50). Similarly, overexpression of *c-Myc* in B-lymphocytes of E μ -*Myc* transgenic mice resulted in cell growth in the absence of cell-cycle progression. Important information is accumulating as to how *c-Myc* may be mediating effects on cell growth. RNA polymerase III (pol III) is involved in the generation of transfer RNA and 5S ribosomal RNA required for protein synthesis in growing cells and is activated by *c-Myc* via binding to TFIIB, a pol III-specific general transcription factor (51). In fact, recent studies suggest that *c-Myc* may regulate the activity of all three known nuclear RNA polymerases (52). It is indeed plausible that *c-Myc*'s role in regulating cell proliferation could at least in part be mediated through its effects on ribosomal biogenesis and cell growth (reviewed in ref. 53).

Insights into how *c-Myc* might promote cell proliferation have resulted from a number of important studies revealing *c-Myc*'s ability to activate or repress target genes involved in cell-cycle progression (see Fig. 3A).

G₁–S progression of eukaryotic cells is controlled by the activities of the cyclin-dependent kinase (CDK) complexes, cyclin D–CDK4 and cyclin E–CDK2. *c-Myc* induces cyclin E–CDK2 activity early in the G₁ phase of the cell cycle, which is regarded as an essential event in *c-Myc*-induced G₁–S progression (54–55). But how does *c-Myc* activate cyclin E–CDK2? It was recently shown that *CCND2* (which encodes cyclin D2) and *CDK4* are direct target genes of *c-Myc* (56,57). Expression of *CCND2* and *CDK4* leads to sequestration of the CDK inhibitor KIP1 (also known as p27) in cyclin D2–CDK4 complexes. The subsequent degradation of KIP1 has recently been shown to involve two other *c-Myc* target genes, *CUL-1* and *CKS* (58). By preventing the binding of KIP1 to cyclin E–CDK2 complexes, *c-Myc* allows inhibitor-free cyclin E–CDK2 complexes to become accessible to phosphorylation by cyclin activating kinase (CAK) (59,60). Increased CDK2 and CDK4 activities would result in

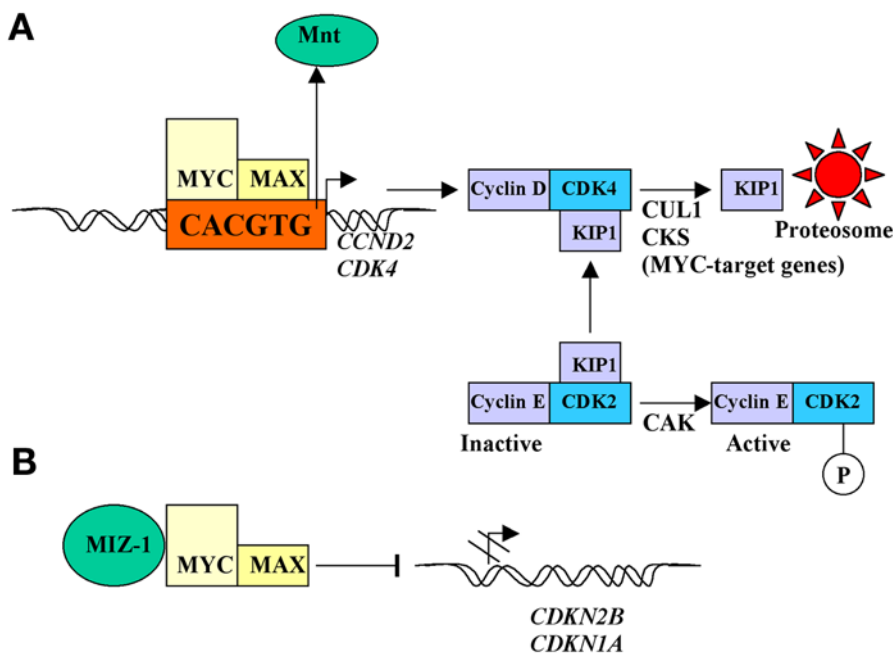


Fig. 3. c-Myc promotes G1–S progression through gene activation and repression. **(A)** c-Myc-Max heterodimers activate target genes, cyclin D2 and cyclin-dependent kinase 4 (Cdk4), which leads to sequestration of Cdk inhibitor KIP1 (p27) in cyclin D2-Cdk4 complexes. Subsequent degradation of KIP1 involves two further c-Myc-target genes, Cul-1 and Cks. In so doing, KIP1 is not available to bind to and inhibit cyclin E-Cdk2 complexes thereby allowing cyclin E-Cdk2 to be phosphorylated by cyclin activating kinase (CAK). **(B)** c-Myc-Max heterodimers repress Cdk inhibitors, INK4B (p15) and WAF1 (p21), which are involved in cell cycle arrest. By interacting with transcription factors, Miz-1 (and/or Sp1), c-Myc-Max prevents transactivation of INK4B (CDKN2B) and WAF1 (CDKN1A), the latter and Inr pathway being predominantly regulated by Miz-1.

Rb hyperphosphorylation and subsequent release of E2F from Rb. Although cyclin D2 is known to be an essential downstream effector of c-Myc in promoting cell proliferation, D cyclins do not appear to be required for c-Myc-induced apoptosis, at least in vitro (59), indicating that these two major functions of c-Myc—proliferation and apoptosis—may involve, at least in part, distinct sets of downstream mediators. It will be interesting to see if this is the case in vivo.

Recent studies support the idea that c-Myc may also exert important influences on the cell cycle by repressing genes, such as the CDK inhibitors INK4B and WAF1 (also known as P15 and P21, respectively)—that are involved in cell-cycle arrest, through the c-Myc-Max heterodimer interacting with positively acting transcription factors such as Miz-1 and SP1 (see Fig. 3B). The interaction of c-Myc-Max with Miz-1 blocks the association of Miz-1 with its own coactivator (P300 protein), with the subsequent down-regulation of INK4B (60) and WAF1 (61).

Much is still being learnt about repression of various genes by c-Myc and this area will undoubtedly be an increasingly interesting one for future studies and therapeutic developments (62–68).

2.4. *c-Myc and Cell Differentiation—Role of the Mad Protein Family*

Activation of c-Myc and subsequent cell-cycle entry is generally incompatible with terminal differentiation. However, the ability of c-Myc to block cell differentiation can be uncoupled from its ability to drive cell proliferation (69,70). Numerous studies have highlighted the importance of the c-Myc/Max/Mad network in regulating cell proliferation and differentiation (71,72). Several lines of evidence support the view that the Mad/Mxi1 bHLH-LZ transcription factor proteins antagonize c-Myc function, for example, ectopic expression of Mad/Mxi1 proteins leads to growth arrest, and suppresses c-Myc-dependent transformation, and growth of tumors (72).

In general, expression of different members of the Mad/Mxi1 protein family coincides with downregulation of c-Myc expression and cells begin to exit the cell cycle and acquire a terminally differentiated phenotype, although there are some exceptions (73–75). In general, c-Myc protein is readily detected in immature proliferating cells, whereas Mad proteins are restricted to post-mitotic differentiating cells (76–78). Whether down-regulation of c-Myc, however, is the trigger for differentiation or a consequence of this cell fate is still unclear. Intriguingly, some studies indicate that onset of differentiation does not always involve cell-cycle arrest; c-Myc may play a role in advancing cells along pathways of epidermal and hematopoietic differentiation (79–81). Whether an increase in cell growth and metabolism induced by c-Myc is important for lineage commitment awaits further investigation.

Mad/Mxi proteins heterodimerize with Max and subsequently repress transcription by recruiting a chromatin-modifying corepressor complex to E-box sites on the same target genes as c-Myc/Max, such that c-Myc-Max complexes can no longer activate its target genes. Mad proteins contain an N-terminal repression domain that associates directly with the corepressor SIN3 (82,83), which in turn binds the class I histone deacetylases HDAC1 and HDAC2. This association suggests that Mad-Max recruits HDACs to its specific target DNA to result in local histone deacetylation within nucleosomes, thereby decreasing the accessibility of DNA to transactivation factors. Recent results from the laboratory of Bob Eisenmann have suggested that the situation may be more complex than previously appreciated; c-Myc and Mad, although possessing identical *in vitro* DNA-binding specificities, do not have an identical set of target genes *in vivo*. In particular, apoptosis is one biological outcome in which the transcriptional effects of c-Myc are not directly antagonized by those of Mad (84). The importance of tight control over c-Myc activity by members of the Mad family, is emphasized by the phenotypes of *Mad1* and *Mxi1* knockout mice (72).

Recent studies have suggested that c-Myc activity may play an important part in maintaining stem cell pluripotency and self-renewal (85). Transcriptional control by LIF and suppression of T58 phosphorylation are crucial for regulation of c-Myc activity in ES cells and therefore in promoting self-renewal. Conversely the c-Myc antagonist Mad and the CDKI, p27 can antagonise this action in hematopoietic stem cells (86,87).

2.5. *c-Myc and Apoptosis*

Putative cancer cells must avoid apoptosis in order for tumors to arise—the net expansion of a clone of transformed cells is achieved by an increased proliferative index and by a decreased apoptotic rate. In the early 1990s, several laboratories made an intriguing discovery: oncoproteins such as c-Myc and the adenovirus E1A—both potent

inducers of cell proliferation—were shown to possess apoptotic activity (88–90). The most widely held view of oncoprotein-induced apoptosis is that the induction of cell-cycle entry “sensitizes” the cell to apoptosis: cell proliferative and apoptotic pathways are coupled. However, the apoptotic pathway is suppressed so long as appropriate survival factors deliver antiapoptotic signals. Given this, the predominant outcome of these contradictory processes will depend on the availability of survival factors.

Since these early experiments, other promoters of cell proliferation (e.g., E2F) have been found to possess proapoptotic activity (91–93). The notion that cells acquiring growth-deregulating mutations *in vivo* possess an “in-built” tumor suppressor function, which hinders expansion of potentially malignant cells, is a fascinating one. Such a cell population would be unable to outgrow its environment unless apoptosis was inhibited. Indirect evidence supports this idea, as shown by the dramatic synergy between oncoproteins such as *c-Myc* and mechanisms that suppress apoptosis, for example overexpression of antiapoptotic proteins such as Bcl-2 or Bcl-X_L, or loss of ARF or P53 tumor suppressors (94–99). Interestingly, stimulation of apoptosis by *c-Myc* may not invariably be a direct effect linked to cell cycling but can also arise through indirect actions culminating in DNA damage. Thus, recent data *in vitro* links *c-Myc* to the accumulation of reactive oxygen species (ROS) via E2F1-mediated inhibition of NFκB, though the consequences, either apoptosis or growth arrest, may be critically dependent on cell type (100,101).

At least two separate pathways are involved in the induction of apoptosis: an intrinsic pathway regulated by various factors such as DNA damage, stress, and imbalances between growth promoting and survival promoting factors that act through mitochondrial permeability, and an extrinsic pathway, utilized, for example, by cytotoxic T cells involving ligation of cell surface death receptors (e.g., Fas and TNFR) and activation of the death inducing signaling complex (DISC). The intrinsic pathway involves activation of the apical procaspase-9, whereas the extrinsic pathway involves activation of procaspase-8. This separation of pathways is complicated, however, by the requirement in some cells for co-opting of the intrinsic pathway by caspase-8 mediated activation of the BH3-only protein Bid. Various proteins regulate these apoptotic pathways, prominent amongst which are the anti- and proapoptotic members of the Bcl-2 family, and the inhibitors of apoptosis (IAPs), such as XIAP. Both pathways have been implicated in mediating the action of *c-Myc* on apoptosis in various studies. A summary of the various mechanisms implicated in *c-Myc*-induced apoptosis is shown (*see* Fig. 4). However, the relative contributions of these mechanisms for a given tissue or cell-type or critically within an individual developing tumor are generally not well known.

How does the *c-Myc* oncoprotein induce or “sensitize” cells to apoptosis? Most of the key experiments used to answer this question have come from cell culture. Although much of the apoptotic machinery has been identified, we are only beginning to define the mechanisms by which *c-Myc* engages such machinery. As is often the case for cell signalling events, there is not just one mechanism by which *c-Myc* engages or activates the apoptotic machinery. The mechanism chosen most likely depends on factors, such as, cell type, signals received from the cells environment, or whether the cell has acquired DNA damage or not. Expression of *c-Myc* sensitizes cells to a wide range of pro-apoptotic stimuli—such as hypoxia, DNA damage and depleted survival factors (8–91)—as well as enhancing sensitivity to signalling through the CD95 (102), TNF

(103), and TRAIL (104) death receptors. In fact, it has recently been shown that c-Myc can lead to down-regulation of the inhibitor of caspase activation, FLICE inhibitory protein (FLIP). FLIP is an inhibitor of the extrinsic pathway, which normally competes with caspase-8 for binding to the DISC- releasing FLIP inhibition may at least in part explain the ability of c-Myc to sensitize cells to death receptor stimuli (105).

Apoptosis can be activated through ligation of cell-surface death-receptors such as Fas/CD95. In some cell types, this alone is sufficient to trigger the downstream caspases and eventual cell destruction. However, in other cells, the death-receptor-induced apoptosis also requires the mitochondrial pathway. c-Myc sensitizes cells to death-receptor signaling by recruiting the mitochondrial pathway.

First insight into c-Myc's role in apoptosis came from studies showing that c-Myc could induce the release of cytochrome *c* from the mitochondria during apoptosis, and that ectopic addition of cytochrome *c* sensitized cells to undergoing apoptosis (106). Once released into the cytoplasm, cytochrome *c* associates with another protein called apoptotic protease-activating factor 1 (APAF-1) to create the apoptosome, which acts as a scaffold for activating procaspase-9 (107). In the presence of cytochrome *c* and either ATP or ADP, caspase-9 is autocatalytically activated, which then activates the downstream caspase effector cascade involving caspases-2,-3,-6,-7,-8, and -10, which break up the cell's components leading to demise of the cell.

mediated gene repression, resulting in increased apoptosis. This feedback mechanism operates independently of p53 (b). In some tumor models, deregulated expression of the inhibitor of ARF, Bmi-1 can co-operate with Myc in tumorigenesis. Ligation of death receptor, CD95/Fas, triggers association of the intracellular adaptor protein FADD with the CD95 receptor and form the DISC (c). FADD then recruits pro-caspase 8 resulting in autoactivation of the pro-caspase, which cleaves and activates executioner caspases. Activation of caspase-8 may be negatively regulated by FLIP, which competes for binding to the DISC. Caspase-8 may also activate the proapoptotic protein, Bid, which may promote MOMP, thereby linking the extrinsic and intrinsic pathways of apoptosis. Recently c-Myc has been reported to mediate apoptosis in some cells by a mechanism involving generation of ROS and suppression of the survival promoting activities of NF- κ B (d). In some cell types Myc can induce expression of the BH3-only pro-apoptotic protein Bim and suppress expression of the antiapoptotic proteins Bcl2 and BclxL (e). c-Myc may increase expression of the p53 family member p73, which might act to direct p53 itself towards proapoptotic genes (f). One question that remains unanswered is how activation of oncogenes such as c-Myc results in expression of ARF, which is not usually activated during normal cell replication. One possibility is through inhibition of proteins like Bmi-1 (g), though this is speculative. Lastly, the inhibitor of caspase activation, FLICE inhibitory protein (FLIP) is transcriptionally repressed by c-Myc, providing a mechanism whereby c-myc could sensitise cells to a wide-range of death receptor stimuli (h). Survival signals that serve to block c-Myc-induced apoptosis (i), include signalling via the IGF1 receptor and PI3 kinase (PI3K) or activated Ras, which can lead to activation of AKT serine/threonine kinase and subsequent phosphorylation of proapoptotic protein Bad. Phosphorylated Bad is sequestered and inactivated by cytosolic 14-3-3 proteins. This pathway may also block apoptosis in other ways. Akt also activates mTOR and it's downstream target the elongation factor eIF-4E. In at least one model expression of eIF-4E could block c-Myc induced apoptosis and cooperate with c-Myc in tumorigenesis. Anti-apoptotic proteins, such as Bcl-2 and Bcl-x_L, reside in the outer mitochondrial membrane and block cytochrome *c* release possibly through sequestration of Bax. The PTEN tumor suppressor acts as a negatively regulator of Akt activation.

c-Myc can also promote apoptosis through its effect on the expression of members of the Bcl-2 family, which are discussed elsewhere. For example, c-Myc represses expression of bcl-2 and bcl-xL, both of which are antiapoptotic proteins, but induces expression of proapoptotic members, such as Bim. This has the effect of permeabilizing the mitochondria to release cytochrome c and other proapoptotic factors, which ultimately lead to activation of caspases.

The Bcl-2 family proteins have recently emerged as fundamental regulators of mitochondrial outer membrane permeabilization (MOMP) necessary for cytochrome c release (108). Although the precise mechanism involved in c-Myc-mediated cytochrome c release is presently unclear, it may involve activation of the proapoptotic protein Bax (109,110), as well as potentially upregulating mitochondrial genes, including cytochrome c, by the transcription factor nuclear respiratory factor-1 (NRF-1), a potential c-Myc-target gene (111). During apoptosis, Bax (normally an inactive monomer) can be induced to oligomerize and migrate from the cytoplasm to the mitochondria by various BH3-only proteins (those Bcl2 family members containing only the single Bcl2 homology-3 domain), such as Bid (112–114). Once inserted into the outer mitochondrial membrane, Bax induces cytochrome c release by the creation or alteration of membrane pores (115–117). Recent studies suggest that c-Myc might activate Bax at least in part by fostering ASK1 activation in the P38 signaling cascade (118). Importantly, mutational inactivation of *Bax* is found to occur during evolution of human colon tumors (119). Antiapoptotic proteins such as Bcl-2 and Bcl-X_L suppress apoptosis by blocking MOMP possibly through sequestration of activated Bax and so preventing cytochrome c release and/or binding to APAF-1 to prevent it from activating caspase-9. In this scenario, the balance of anti- and proapoptotic molecules present within a c-Myc-activated cell would determine whether it lives or dies.

The survival factor, IGF-1, has been shown to inhibit c-MYC-induced apoptosis in vitro by blocking cytochrome c release from the mitochondria (120). Survival signals mediated via the IGF-1 receptor or activated Ras (see Fig. 4) can lead to activation of the AKT/PKB serine/threonine kinase (121). Activated AKT then phosphorylates the pro-apoptotic BH3-only protein BAD resulting in its sequestration and inactivation by the cytosolic 14-3-3 proteins (122). Referring back to the initial experiments in vitro, in which cells with deregulated expression of c-Myc die by apoptosis when grown in low serum, it becomes clear how growth factor signaling is able to regulate apoptosis. Given the importance of such growth factors in determining the survival or death of a cell, it is not surprising that elevated signaling through the IGF-1 pathway occurs in many tumors. Similarly, genetic mutations that activate the PI3K pathway dramatically collaborate with c-Myc during tumor progression.

Another possible mechanism that links c-Myc and apoptosis is through its indirect activation of P53 via p19^{ARF} (ARF) (123). The ARF tumor suppressor protein acts in a checkpoint that protects against unscheduled cellular proliferation in response to oncogenic signaling. Deregulated expression of c-Myc induces ARF expression and apoptosis through the ARF-Mdm2-p53 axis. However, ARF also prevents hyperproliferation and transformation caused by c-Myc and enhances c-Myc-induced apoptosis independently of p53 (124) (see Fig. 5).

The tumor suppressor, p53, is a master regulator of cell proliferation, and can induce apoptosis, senescence, or DNA repair in response to a variety of cellular stresses, including

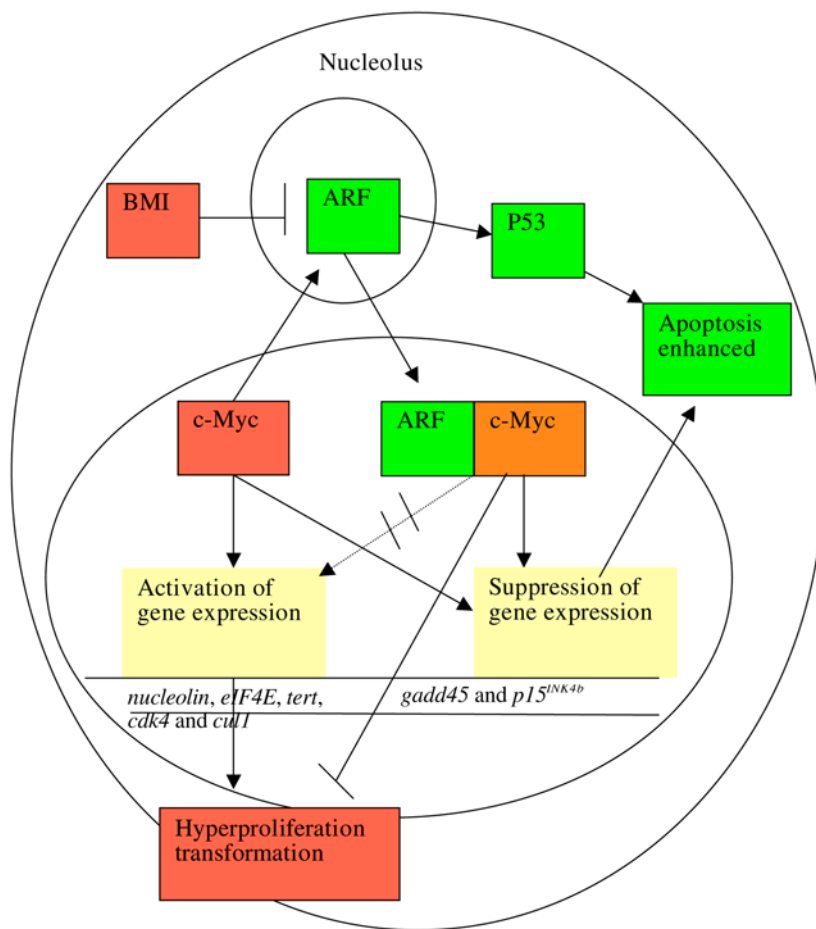


Fig. 5. ARF can promote c-Myc induced apoptosis directly or indirectly via p53. In the presence of c-Myc activation, ARF relocates from the nucleolus to the nucleus. ARF may bind to c-Myc thus preventing c-Myc transcriptional activation, but not the transcriptional repression. By this means, ARF may augment c-Myc induced apoptosis and block hyperproliferation-resisting tumorigenesis. ARF may also cooperate with c-Myc in inducing apoptosis via induction of p53. BMI may cooperate with c-Myc in tumors models in part via inhibiting ARF transcription.

DNA damage, hypoxia, and nutrient deprivation. The importance of p19^{ARF} in c-MYC-induced apoptosis in vivo was underscored in genetically altered mice in which expression of *c-MYC* was targeted to B-lymphocytes. When these mice were cross-bred with another strain in which p19^{ARF} was disrupted, Myc-induced lymphoma development was dramatically accelerated. This outcome, similar to that seen when p53 is disrupted, shows that c-MYC strongly collaborates with loss of p53 or p19^{ARF} in murine lymphomagenesis presumably by inhibiting c-MYC-induced apoptosis (extrapolating from in vitro experiments). In a similar fashion, deregulated expression of *Bmi1* oncogene (which encodes a polycomb group protein), accelerates c-Myc-induced lymphomas by inhibiting expression from the *Ink4a* locus, which encodes Arf and p16^{INK4A} (see Chapter 7). We now know that P53 activates many proapoptotic proteins, such as Bax, Apaf1, Noxa, and

puma, which are involved in cytochrome *c* release from the mitochondria and/or the activation of caspases—enzymes responsible for the destruction of the cell.

Tiam1, a specific guanine nucleotide exchange factor of Rac1 binds to the c-Myc box II of c-Myc and modulates several of its biological functions. Overexpression of Tiam1 inhibits c-Myc apoptotic activity (125). Recently the MBIII region has also been implicated in apoptosis.

It is clear that the favoured mechanism for c-Myc-induced apoptosis may be dictated by cell type as well as by tissue location and moreover modified by the presence or absence of additional mutations in other pro- and antiapoptotic genes.

2.6. c-Myc the Model “Super-Competitor”

Recent studies of the phenomenon of cell competition in *Drosophila* have produced some intriguing findings that may have implications in human cancer (126–128). These recent studies in *drosophila* demonstrate that Myc may have another role in regulation of cell numbers beyond its ability to regulate expression of growth-promoting genes. The ability of Myc to induce apoptosis cell-autonomously has been extensively discussed already. However, it now seems that Myc may also induce apoptosis in neighboring cells—a non-cell-autonomous response. Thus, two recent papers in *Cell* have shown that cells with higher levels of Myc expression not only outgrow their neighbors with lower levels of Myc, perhaps not surprising, but directly lead to apoptosis in the cells with lower Myc expression. Cells with high levels of Myc expression can act as super-competitors that are capable of both out-growing and inducing death in nearby cells with lower Myc levels.

One likely explanation is that such cells are more efficient at accessing survival signals and/or growth factors in the microenvironment than cells with lower levels of Myc. Interestingly, other growth-promoting pathways that have been examined do not cause apoptosis in neighboring cells. Given that c-Myc is well-known to regulate multiple genes involved in biosynthesis cell growth and replication, it can be postulated that oncogenic *Myc* would enable cells to grow and clonally expand at the expense of their wild-type neighbors, which may even be killed as a consequence.

In order to further illuminate the complex interactions surrounding c-Myc and apoptosis, a role so sensitive to tissue location in vivo, we eagerly await the outcome of further studies in which apoptotic pathways are selectively manipulated in mouse mutants or by employing new pharmacological tools and phenotype determined in the all important context of the intact organism.

3. c-Myc, Evasion of Apoptosis and Cancer

Whereas regulated c-Myc expression occurs during normal cell proliferation in response to extracellular signals, such as ligation of growth factor receptors, constitutive or deregulated expression of c-Myc is often associated with tumor-derived cells. Unlike normal cells, tumor cells no longer require exogenous mitogenic stimulation from their tissue microenvironment in order to proliferate. This poses a danger to the host, as the normal restraints that exist in a given tissue to limit uncontrolled cell growth are disrupted.

Early in vitro experiments showed that constitutive overexpression of c-Myc can immortalize rat fibroblasts and prevent withdrawal from the cell cycle (128,129). Although oncogenic activation of c-Myc alone causes uncontrolled proliferation in

vitro, cellular transformation in vitro seemed to require additional oncogenic lesions (130–135).

Deregulated c-Myc expression is often associated with aggressive, poorly differentiated tumors; however, given that most human tumors are quite advanced at the time of discovery—often possessing many genetic alterations—it is difficult to ascertain at which stage of tumor progression, oncogenic *c-Myc* activation occurred. This is an important point if we wish to establish the role of c-Myc in the initiation of a tumor, or indeed as a therapeutic target at later stages of progression. It is assumed from the majority of in vitro and in vivo data that the predominant role of deregulated c-Myc in tumor initiation/progression in vivo is through uncontrolled cell proliferation concomitant with loss of terminal differentiation (in most cases). Although this may be part of the picture, it is conceivable that there are other attributes afforded to c-Myc (e.g., the formation of new blood vasculature in the growing tissue mass) that are impossible to determine in the culture dish and in conventional transgenic systems.

Studies employing conventional c-Myc transgenic mice, in which the oncogene is constitutively expressed in a given cell type by means of a tissue-specific promoter, have supported the view that deregulated c-Myc, as an initial event, is important for the formation of certain cancers, albeit with a long latency (94,95). However, it has been difficult to directly observe the proposed tumor suppressor function of c-Myc (apoptosis) because the oncoprotein is expressed through much of development and thereafter, and would be expected to obliterate the very cells in which it induced apoptosis.

Acceleration of tumorigenesis in transgenic mice is observed when apoptosis is suppressed. However, it is unclear whether exacerbation of c-Myc-induced tumorigenesis in these models is a direct consequence of antagonizing c-Myc-induced apoptosis or by nonspecific protection of cells from the apoptotic influence of genotoxic and other stresses. Indeed, the physiological relevance of c-Myc-induced apoptosis as a tumor suppressive mechanism in vivo has, until recently, been unclear. Moreover, as most human cancers are precipitated by somatic mutations that arise sporadically in adult tissues concerns were raised as to whether the early activation of the onco-transgene in mice accurately reflected the human disease.

These issues led to the development of regulatable transgenic mice in which oncogene expression is controlled within the target tissue. Although the widespread expression of given onco-transgenes (in all cells of the target tissue) does not precisely recapitulate the process during development of sporadic tumors, the ability to activate oncogenes at will in the adult provides a significant advance in the generation of physiological cancer models and an unprecedented potential to validate new drug targets by subsequently deactivating the oncoprotein (see Fig. 6).

A conditional transgenic expression system (c-MycER^{TAM}) was employed to investigate the effect of “switching on” c-Myc activation in distinct tissues of adult transgenic mice (14,15,135–137). Directed expression of the conditional form of the c-Myc oncoprotein (c-MycER^{TAM}) to the suprabasal epidermis or pancreatic islet β -cells was achieved by placing *c-MycER^{TAM}* cDNA under the control of an involucrin promoter (15,137) or an insulin promoter (14,137), respectively.

The differences in cell behavior in these two mouse models following c-Myc activation was striking. In the suprabasal epidermis (15), c-Myc activation is sufficient to induce cell-cycle entry of postmitotic keratinocytes concomitant with impaired differentiation,

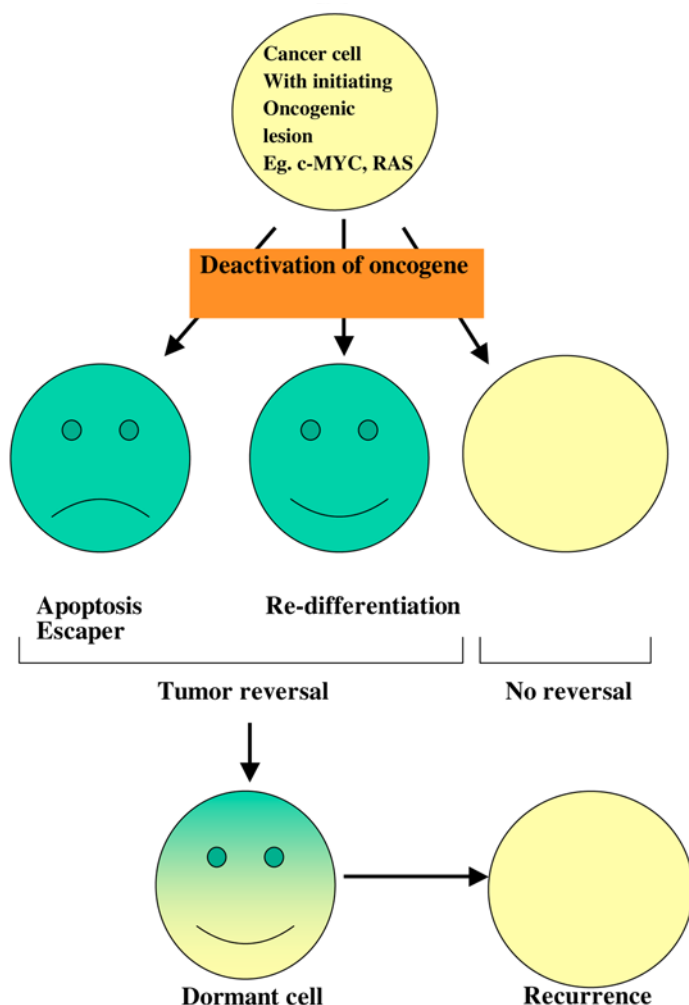


Fig. 6. Tumor regression following de-activation of initiating oncogenic lesion. Various transgenic mouse models of tumorigenesis have shown that subsequent deactivation of the initial oncogenic lesion leads to rapid and complete regression of most neoplastic lesions. Mechanisms of tumor regression include tumor cell apoptosis and/or differentiation and re-establishment of cell–cell contacts. Onset of vasculature collapse within tumor masses occurs rapidly which may also contribute to tumor cell apoptosis. In some cases, a subset of tumors escape reversal (“escapers”) and thus are no longer maintained by the initiating oncogenic lesion. In these cases, it is likely that additional oncogenic lesions have occurred. Finally, a number of cells may remain dormant in an apparently regressed tumor that can after even along latency give rise to a recurrent tumor.

culminating in epidermal hyperplasia with areas of focal dysplasia and papillomatous lesions resembling the human premalignant skin lesions known as actinic keratosis—a precursor of squamous cell carcinoma. A notable feature was the widespread induction of angiogenesis, which argues against the necessity of a specific “angiogenic switch” in a subpopulation of tumor cells ([138](#)). Instead, the angiogenesis observed may merely reflect the dramatic increase in the number of vascular endothelial growth factor (VEGF)-secreting keratinocytes as a consequence of c-Myc ([138,138a](#)), although other angiogenic factors have yet to be investigated. Importantly, c-Myc has also been shown to be angiogenic in

multiple cell types both through its repression of the angiogenesis inhibitor Thrombospondin-1 (139–142) and its induction of VEGF (15,143,144). Recently, it has been shown that Myc may lie downstream of Ras signaling in the promotion of angiogenesis at least in part by inhibiting Thrombospondin 1 (142). The key observation was the obvious lack of c-Myc-induced apoptosis in epidermal keratinocytes in vivo, despite the potent induction of apoptosis by c-Myc in vitro in isolated serum-deprived keratinocytes from the same transgenic mice. In this tissue proliferation is the predominant outcome over apoptosis. One can speculate that in this tissue the oncogenic potential of c-Myc is buffered by the continued differentiation and migration of keratinocytes to the skin surface where they become keratinized and then shed.

In contrast to skin, the predominant effect of activating c-Myc in pancreatic β -cells of adult transgenic mice (14) is apoptosis, and not proliferation. Thus, although activation of c-MycER^{TAM} is followed by widespread cell-cycle entry, in complete contrast to epidermal keratinocytes in vivo, c-Myc activation soon triggers rapid apoptosis of islet β -cells. Such β -cell apoptosis overwhelms any concomitant proliferation, resulting in islet involution and onset of diabetes within 9 d. The acute sensitivity to induction of apoptosis by c-Myc suggests that β -cells are only modestly buffered against cell death by survival signals or intrinsic antiapoptotic mechanisms, such as Bcl-2/Bcl-x_L expression, in vivo. The fact that the innate proapoptotic growth-suppressive potential of c-Myc dominates its growth-promoting action strongly indicates that, in this tissue, c-Myc acts as its own “tumor suppressor.”

However, suppression of apoptosis (by coexpressing Bcl-X_L), exposes multiple innate oncogenic properties of c-Myc that are sufficient to trigger immediate carcinogenic progression (14). These cellular aspects include immediate and sustained b-cell proliferation, which results in inexorable islet expansion concomitant with loss of differentiation (down-regulation of insulin and other differentiation markers), angiogenesis, loss of cell–cell contacts resulting from loss of E-cadherin, and local invasion of b-cells into surrounding exocrine pancreas. In contrast, with the RIP1-Tag2 model of b-cell carcinogenesis (145), tumor progression is ubiquitous and rapid (2 wk) with multiple diverse aspects emerging concurrently. The dramatic and immediate oncogenic progression observed in the majority of islets in vivo strongly supported the at that time unorthodox notion that complex neoplastic phenomena involving the tumor cell and its interactions with normal surrounding tissues, may both be induced and maintained by a very limited platform of interdependent pleiotropic lesions.

3.1. Tumor Cell Apoptosis and Regression Following Oncogene Deactivation

Conditional systems to induce oncogenes in a tissue-specific and time-dependent manner also enable the study of tumor reversal, following deactivation of the oncogene (reviewed in refs. 146,147). Given the importance of oncogene activation in human cancers, specific targeting of oncogenic pathways provides a potentially effective therapeutic strategy, which can be tested in such models. For example, targeting of the HER2/Neu receptor tyrosine kinase (which is overexpressed in up to 30% of primary human breast cancers) with the neutralizing antibody Trastuzumab, has been used successfully in clinical trials, in combination with other agents, to slow disease progression (*see ref. 148 and references therein*). Similarly, patients with chronic myelogenous leukaemia (CML) have been effectively treated with the ABL kinase inhibitor, imatinib (Gleevec), inducing clinical remission whilst in the CML phase (149,150).

Previously, we have shown that sustained c-Myc inactivation in locally invasive pancreatic islet tumors (induced by c-Myc activation in β -cells on a background of Bcl-x_L overexpression) induced β -cell growth arrest and re-differentiation into mature β -cells, accompanied by the collapse of tumor vasculature and tumor cell mass resulting from apoptosis, despite the constitutive expression of Bcl-x_L in tumor cells (14). Similarly, in premalignant skin epidermal tumors (papillomatosis) induced following activation of c-Myc alone, skin lesions completely regressed within 4 wk after sustained inactivation of c-Myc (15). However, given the continual outward migration and shedding of growth-arrested and redifferentiated keratinocytes from the skin surface, it was not established whether this action alone was responsible for the removal of skin tumor cells or if apoptosis also played a part.

Tumor regression has now been observed in numerous other mouse cancer models (Table 1), following tagetting of various oncogenes. In fact, several studies, using conditional mouse models of various cancers, have shown unexpectedly, that inactivation of the initiating oncogene is sufficient not only for reversal of the primary tumor but also of invasive and metastatic lesions, many of which contain multiple genetic and epigenetic alterations (14,15,151–169). The tumor regression observed in many of these tumor models following sustained oncogene inactivation, provides a powerful platform on which to build a deeper understanding of fundamental tumor biology and with which to preclinically evaluate novel therapeutics to target specific genes. However, as discussed later, there are some instances when more advanced tumors do not fully regress and further insight into the mechanisms of this divergence is required.

3.2. Epigenetic Context, Apoptosis, and Tumor Regression

A recent study has shown that brief inactivation (10 d) of c-Myc was sufficient for the sustained regression of c-Myc induced invasive osteogenic sarcomas in transgenic mice (170); subsequent re-activation of c-Myc led to extensive apoptosis rather than restoration of the neoplastic phenotype. Possible explanations for this outcome include changes in epigenetic context that may have occurred within the cell type, that is, presumably between the immature cell in which c-Myc was originally activated and the more differentiated cell resulting from subsequent (brief) inactivation of c-Myc. In this tumor model, although c-Myc expression is initiated in immature osteoblasts during embryogenesis, subsequent inactivation of c-Myc in osteogenic sarcoma cells induces differentiation into mature osteocytes. Therefore, reactivation of c-Myc now takes place in a different cellular context and induces apoptosis rather than neoplastic progression. However, irrespective of the actual underlying mechanisms, these intriguing findings suggest the novel possibility of employing transient inactivation of c-Myc as a therapeutic strategy in certain cancers, thus limiting potential toxic effects resulting from prolonged therapeutic inactivation (146,147). However, to date this appears to be a unique feature of this model and sustained regression of tumors originating in different tissues and under differing circumstances following transient c-Myc inactivation has not been shown.

Thus, inactivating c-Myc transiently in skin keratinocytes or pancreatic β -cells does not lead to sustained regression. Rather, and in contrast to the osteogenic sarcoma model, reactivating c-Myc in islet tumors does not lead to accelerated β -cell apoptosis, but rather

Table 1
Use of Regulatable Transgenic Mouse Models of Cancer to Test Tumor Regression

Initial oncogenic lesion	Target tissue/cell type	Phenotype	De-activating initiating oncogene	Ref.
SV40 T antigen	Embryonic submandibular gland	(i) Atypical cells (4-wk-old) (ii) Transformed ductal cells (7-mo old)	(i) Full phenotype reversal (ii) Not reversed	216
H-Ras (+Ink4a ^{-/-})	Melanocytes	Melanoma	Rapid tumor reversal Larger tumors (20%) not reversed	217
c-Myc	Embryonic hematopoietic cells	T cell lymphomas/ acute myeloid leukaemias (5-mo old)	Rapid tumor reversal 10% tumor relapse	151
c-Myc	Adult suprabasal epidermis (keratinocytes)	Papillomatosis (carcinoma <i>in situ</i>)	Rapid and complete tumor reversal	15
c-Myc	Adult suprabasal epidermis (keratinocytes)	Papillomatosis (carcinoma <i>in situ</i>)	Tumor recurrence after transient c-Myc inactivation	137
c-Myc	Adult suprabasal epidermis (keratinocytes)	Papillomatosis (carcinoma <i>in situ</i>)	Growth arrest after transient c-Myc inactivation	138a
Bcr-Abl1	B-cells	Acute B-cell leukaemia	Complete tumor reversal	218
c-Myc	Mammary epithelium	Invasive mammary adeno-carcinoma	Tumor reversal Subset not reversed (Ras activated)	169
K-Ras	Lung	Adeno-carcinoma	Tumor reversal	219
K-Ras (+p53 ^{-/-} or Ink4a ^{-/-} or ARF ^{-/-})	Lung	Adeno-carcinoma	Rapid and complete tumor reversal	220
c-Myc (+Bcl-xL)	Adult pancreatic islet β -cells	Invasive islet adeno-carcinoma	Rapid and complete tumor reversal	14

(Continued)

Table 1 (Continued)

Initial oncogenic lesion	Target tissue/cell type	Phenotype	De-activating initiating oncogene	Ref.
c-Myc (+Bcl-xL)	Adult pancreatic islet β -cells	Invasive islet adeno-carcinoma	Tumor recurrence after transient c-Myc inactivation	137
c-Myc	Embryonic osteocytes	Malignant osteogenic sarcoma	Rapid and complete tumor reversal even after transient inactivation	170
c-Myc	Hepatocytes	Hepatocarcinoma	Tumor reversal, Dormant tumor cells give rise to recurrent tumors, when Myc is reactivated	172
c-Myc	Lymphocytes	T- and B-cell lymphoma	Reversal	160
Bcl2 (+ c-Myc)	Lymphocytes	Lymphoblastic leukaemia	Apoptosis and Reversal	164
Neu (and MMTV)	Mammary epithelium	Invasive mammary carcinoma	Essentially complete reversal even of metastatic lesions. Eventually, however tumors spontaneously recur	221
Wnt	Mammary epithelium	Invasive mammary carcinoma	Essentially complete reversal even of metastatic lesions. Eventually, however tumors spontaneously recur	158
Gli2	Basal keratinocytes	Basal cell carcinoma	Reversal	222

The potential for tumor regression following deactivation of the initial oncogenic lesion (c-MYC, SV40 T antigen, H-Ras, K-Ras, BCR-ABL, Neu, Wnt and Gli2) has been investigated using several regulatable transgenic mouse models of cancer. In general, the findings listed above have implications for therapy as they indicate that blocking oncogene function, even in advanced tumors, could lead to apoptosis or differentiation of tumor cells. Further to these findings, it was recently shown that transient, rather than sustained, inactivation of c-MYC is sufficient for full reversal of malignant osteogenic sarcoma in transgenic mice. If valid for other cancer models, transient inactivation of oncogenes could provide an effective cancer therapy limiting host cell toxicity. However, it has subsequently been shown in various other tissues, that such transient inactivation is not sufficient for tumor reversal (*see text*). In fact, in some tumors inactivation of the transgene is not followed by complete regression of all tumors or recurrence occurs spontaneously in regressed tumors, suggesting that mutations occur which can make the tumors independent of the initiating lesion.

restores the oncogenic properties of c-Myc, rapidly reinitiating β -cell proliferation, loss of differentiation, loss of E-cadherin, local invasion, and angiogenesis. This occurs despite the re-expression of differentiation markers and the loss of some of the newly acquired vasculature, occurring during the period of c-Myc inactivation. Moreover, as no new β -cells arise, during the period of c-Myc inactivation, replication is likely restored in those same cells that have previously experienced c-Myc activation. Similarly, in epidermis, reactivating c-Myc in suprabasal keratinocytes does not result in apoptosis, which remains confined to the shedding areas of parakeratosis at the skin surface, but restores the papillomatous phenotype, inducing cell proliferation and dysplasia (137). These results are in line with a very elegant recent study in a different system by Shachaf and colleagues, who demonstrate that invasive c-Myc-induced hepatocellular carcinomas regress when c-Myc expression is turned off but, interestingly, some tumor cells remain “dormant” even for prolonged periods and contribute to cancer progression if c-Myc expression is subsequently reinitiated (172). One of the most important aspects of this study was the use of a “lineage tracking” system that unambiguously allowed the researchers to conclude that tumor recurrence derived from cells that had previously been in the regressed cancer rather than de novo from previously normal cells.

Different types of cancers are prevalent in different age groups with the effects of oncogene activation dependent on the developmental stage of the target cell at that time. The biological consequences of activating c-Myc are clearly influenced not only by environment but also by developmental stage. This has been shown in two recent mouse tumor models, mammary gland and liver. C-Myc can inhibit postpartum lactation if activated within a specific 72-hour window during mid-pregnancy, whereas c-Myc activation either prior to or following this 72-h window does not (161). In embryonic and neonatal mice, c-Myc overexpression in the liver immediately results in hyperproliferation and neoplasia, whereas in adult mice c-Myc overexpression induces cell growth and DNA replication but without mitotic cell division, and neoplasia is considerably delayed (163).

Taken together, these findings suggest that a cautious approach is required in considering cancer therapies aimed at transient oncogene inactivation. First, a more comprehensive understanding of the genetic basis and environmental context of any individual tumor would be required in order to predict the likely success of such a treatment schedule. Second, at least under those circumstances where tumor cell differentiation and alteration of epigenetic context would not be predicted to reinstate apoptosis and no alternative mechanism exists for tumor cell removal, sustained inactivation of the offending oncogene would seem the desired therapeutic goal.

3.3. Tumor Regression—Cancer Cell “Escapers” and “Sleepers”

There are a few instances when more advanced tumors do not fully regress (Table 1) and, in these cases, further insight into the mechanisms of this divergence is required. Although it is not clear as to why some tumors escape dependence upon the c-Myc transgene, it has been reasoned that these tumors acquire genetic lesions that in some way substitute for the requirement for c-Myc. Importantly, tumor regression following down-regulation of c-Myc expression coincided with tumor cells differentiating to more mature lymphocytes with the restoration of normal host hematopoiesis, as well as a proportion of tumor cells being eliminated by apoptosis (157). Reversal of c-Myc-induced invasive mammary adenocarcinomas has also been shown in a mouse model, although one subset of tumors failed to reverse and were found to carry additional mutations in

Ras (169). In fact, recent studies have demonstrated that c-Myc may cause genomic instability in some model systems in vivo (173) and in vitro (174,175), which certainly in the long-term could contribute to c-Myc-induced neoplastic progression.

In general, the above findings have implications for therapy as they indicate that blocking oncogene function, even in advanced tumors, could lead to apoptosis or redifferentiation of tumor cells. The potential for tumor regression following deactivation of initial oncogenic lesions (c-Myc, SV40 T antigen, H-Ras, and K-Ras) have provided valuable information and hope for future development of candidate drug molecules (Table 1).

3.4. Activated c-Myc Expression in Human Tumors

Activation of c-Myc, as is the case in many human tumor cells, is defined as deregulation of the normal, highly controlled expression pattern of the *c-Myc* proto-oncogene. In normal dividing cells, c-Myc expression is maintained at a relatively constant intermediate level throughout the cell cycle, whereas in its oncogenic form, c-Myc might be constitutively expressed at levels ranging from moderate to very high (176), and is non-responsive to external signals (4–6). Alternatively, the regulated pattern of c-Myc expression can remain intact, but exceed normal levels of expression for the given cell type.

c-Myc activation can occur by a wide range of direct and indirect mechanisms, such as stabilization of c-Myc mRNA transcripts or through enhanced initiation of translation resulting from mutation of the internal ribosomal entry site (177,178), or indirectly via activation of upstream signaling cascade (179–182). Historically, molecular pathologists have relied on the presence of gross chromosomal abnormalities, such as translocation or amplification, of the *c-Myc* locus to define activation of this oncogene in tumor cells (as is the case in Burkitt's lymphoma and neuroblastoma, respectively). However, this restrictive diagnostic criterion has resulted in an underestimate of the numbers of tumors with deregulated *c-Myc*.

The detection of c-Myc activation in tumor cells now relies on both elevated expression of *c-Myc* mRNA and genetic alterations of the *c-Myc* locus. Advances in technology allow us to evaluate expression levels of *c-Myc* mRNA in tumor cells (precisely excised using laser-capture microdissection) with real-time reverse transcription-polymerase chain reaction (RT-PCR) and expression profiling using microarray technology (183,184). These approaches allow rapid, quantifiable, high-throughput screening of *c-Myc* activation in tumor cells. However, these techniques do not measure levels of c-Myc protein expression, which are largely assayed by immunohistochemical methods. Evidence now indicates that *c-Myc* activation can also occur through stabilization of the c-Myc protein. c-Myc proteins with mutations at or near T58, which abolish phosphorylation at this site, result in inefficient ubiquitination and a stabilization of the protein (185,186).

The fact that c-Myc activation is present in a broad range of human cancers, and is often associated with poor prognosis of the disease, suggests that analysis of c-Myc deregulation in certain tumor types may be used as a diagnostic marker. Moreover, inactivating c-Myc, or downstream targets of c-Myc, may provide important therapeutic targets. Although still in its infancy, various approaches to target c-Myc are presently under investigation (187,188).

4. c-Myc and Diabetes

Diabetes is a serious global health problem affecting 194 million people worldwide and this figure is expected to rise to 370 million by the year 2030. Current preventative and therapeutic strategies are manifestly inadequate, and there is a need for research to develop novel healthcare interventions to address this substantial biomedical challenge. Diabetes is caused by an absolute (type 1) or relative (type 2) deficiency of insulin-producing β cells.

Type 2 diabetes (T2D) constitutes between 85 and 95% of all diabetes and is caused by two distinct but overlapping processes, namely peripheral *insulin resistance* in muscle, fat, and liver and failure of pancreatic β -cells to adequately compensate for this resistance—“ β -cell failure.” β -cell failure encompasses impaired glucose sensing/insulin secretion and loss of β -cell mass (imbalances in β -cell apoptosis and renewal) and is now widely appreciated as the pre-eminent factor in T2D onset and disease progression in man (189).

Comparatively little is understood about the causes of β -cell failure and much less is known about how to prevent or reverse it. Encouraging results with lifestyle intervention and metformin notwithstanding, large studies such as the United Kingdom Prospective Diabetes Study (UKPDS) suggest currently available therapies do not prevent relentless progression of β -cell failure in T2D.

To progress in this key area we urgently need to develop a more complete picture of how key β -cell processes, such as replication, apoptosis, neogenesis, and differentiation, are controlled and how they become derailed in T2D. Moreover, better understanding of β -cell growth and regeneration mechanisms may allow new strategies in the treatment of T2D based on early limitation of β -cell damage and/or restoration of a functional β -cell mass.

4.1. c-Myc Apoptosis, β -Cell Failure and Diabetes

Recent studies by several laboratories, including our own, have provided support for the notion that c-Myc could provide a molecular link between insulin resistance (and accompanying adaptive β -cell growth/replication) and the two major potential contributors to β -cell failure: loss of function and sensitisation of β -cells to apoptosis. c-Myc is clearly of great relevance to β -cells; it is upregulated in and required for replication (G_1/S transition) in essentially all cell types, including β -cells, and is an important contributor to cell growth (size) even where replication is absent; c-Myc has been shown repeatedly to be expressed in β -cells exposed to hyperglycaemia, during increases in β -cell mass in vivo and in vitro and in β -cell-derived neoplasia. In fact, by implication c-Myc will be elevated in β -cells in insulin resistance as an inevitable accompaniment to the adaptive increases in β -cell mass and ultimately also in response to rising blood glucose (189).

In animal models of diabetes, impaired glucose-stimulated insulin secretion (GSIS) is associated with loss of β -cell differentiation markers and of proteins involved in glucose stimulus-secretion coupling; accompanied by β -cell hypertrophy and increased expression of c-Myc (190–193). Recent studies from our own laboratory and that of Susan Bonner-Weir and Gordon Weir have shown that c-Myc is a powerful trigger for β -cell apoptosis as well as loss of differentiation in rodent islets in vivo (14,194). Importantly, c-Myc activity in β cells is enhanced by hyperglycaemia. Given, that c-Myc activation leads to reduced insulin content and also β -cell apoptosis in vivo, (14)

induction of c-Myc by hyperglycemia may contribute to β -cell loss/dysfunction in diabetes (*see Fig. 7*). This is discussed in more detail below

During the progression to diabetes both functional defects and β -cell apoptosis contribute to defective insulin secretion (*189–191*) generally described as β -cell failure. Several potential contributors to this have been identified, including defective insulin/IGF/IRS2 signalling, activated c-Myc, and various extrinsic factors including glucose toxicity, lipotoxicity, various inflammatory cytokines and oxidative stress (*193*). Importantly, recent studies suggest that replicating β -cells may be particularly vulnerable to apoptosis, thus indirectly implicating c-Myc (*193a*).

The notion that c-Myc may be involved in β -cell failure is further supported by studies showing that β -cells also upregulate c-Myc in response to rising blood glucose levels. Even mild hyperglycaemia can adversely affect β -cell phenotype over time and result in progressive loss of β -cell differentiation as evidenced by reduced expression of key transcription factors (e.g., Pdx-1, BETA2/NeuroD) and genes involved in glucose sensing (GLUT-2 and glucokinase) (*192,192a*). On the other hand, genes normally suppressed in β -cells (c-Myc and various target genes such as lactate dehydrogenase-A) are increased. Recent studies have helped resolve this complicated interaction- ectopic activation of c-Myc in β -cells results in loss of differentiation markers and also in apoptosis. Thus, a likely model is that β -cells activate c-Myc in response to a need for adaptive growth or later in the process because of rising blood glucose. Elevated c-Myc may then contribute to loss of function and apoptosis of β -cells. Various processes, apart from c-Myc, have been described as underlying glucose toxicity and include altered calcium homeostasis, activation of the extrinsic pathway of apoptosis by cytokines, such as IL-1 β or Fas/Fas-ligand interactions and reactive oxygen species. Intriguingly, c-Myc has been shown to trigger apoptosis through all of these pathways in other systems— including in particular activation of both apical caspases (caspase 8 and 9), Fas/Fas-ligand signalling and reactive oxygen species.

Targeting c-Myc-induced β -cell apoptosis and loss of differentiation would represent an attractive therapeutic option in diabetes. However, the means of preventing apoptosis may need to be carefully approached, given that suppressing c-Myc-induced apoptosis by overexpression of Bcl-X_L in mouse islets *in vivo* rapidly leads to c-Myc-induced beta cell neoplasia, described earlier (*14*). Interestingly, a recent study suggests that the growth effect of Pax4 may be mediated by concurrent activation of both c-Myc and BclxL, and may thus play a role in normal expansion of β -cells (*195*). By implication, it is deregulated and excessive expression of c-Myc and possibly BclxL that is oncogenic, whereas controlled expression, likely in response to normal growth-factor regulation is not.

Various downstream targets and processes of c-Myc may also contribute to β -cell growth, including cyclin D2 and its partner CDK, CDK4 [*196–200*]. Moreover, inactivation of the CDKI, p27 is also able to promote beta cell growth.

5. Therapeutic Targeting of c-Myc

Given the central role of c-Myc in cell proliferation and death and, moreover, in the development and maintenance of many cancers, the potential therapeutic benefits of targeting aspects of c-Myc activity in human cancers are self-evident. Moreover, studies employing c-Myc-directed therapies may also finally help address a central question

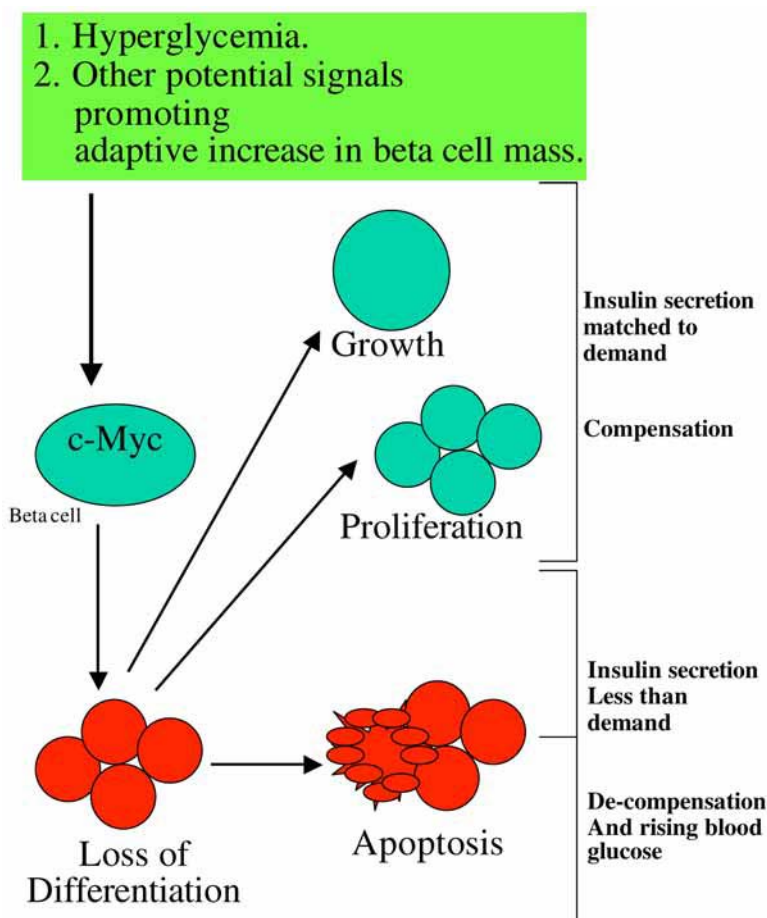


Fig. 7. Potential role of c-Myc in beta cell failure in diabetes. Type 2 diabetes arises or progresses when there is an imbalance between apoptosis and compensatory β -cell growth processes. Hyperglycaemia leads to c-Myc expression in insulin-secreting β -cells of the pancreatic islets. Although, as yet to be confirmed it is highly likely that c-Myc will be upregulated in β -cells induced to grow/replicate as part of normal adaptive growth (e.g., in response to pregnancy) or in response to insulin resistance. In animal models, following c-Myc activation β -cells replicate but undergo concomitant apoptosis. Moreover, even replicating β -cells that are prevented from undergoing apoptosis experience loss of differentiation as evidenced by reduced insulin content, and loss of markers such as Pdx1.

remaining unanswered in human cancer, namely can human tumors be reversed as readily as mouse equivalents by targeting an individual oncogenic lesion.

In order to realize the potential of targeting c-Myc in the treatment of cancer, drugs will have to be designed to manipulate specific c-Myc actions, such as cell-cycle entry, dedifferentiation, or apoptosis. It is likely that this will require the identification of druggable targets downstream of c-Myc activation. Despite recent advances in the identification of multiple c-Myc-target genes by employing powerful analytical techniques such as gene microarrays most of these studies have relied on the study of cell lines or tissues in vitro (201–204), or during c-Myc-induced lymphomagenesis in chicken following

transplantation of c-Myc infected embryonic bursal cells (205). Interestingly, the latter study suggested that the level of c-Myc over-expression had a major impact on the numbers of genes regulated. Given the critical dependence of c-Myc-induced phenotype on tissue location *in vivo* we still do not know enough about the key divergence in c-Myc-targets under conditions, which favor the opposing potential outcomes of c-Myc-activation, namely either apoptosis or proliferation. However, there is considerable reason for optimism. Proof of hypothesis exists already, for example, at least in some situations, Cyclin D is essential for c-Myc-induced proliferation but not for c-Myc-induced apoptosis (*in vitro*), and the same may well apply to other genes/proteins. Studies in which the immediate and sequential evolution of changes in gene/protein expression can be tracked and correlated with the emergence of key cancer phenotypes *in vivo* are now required. To undertake this will involve the application of powerful new advances in conditional mutagenesis alongside analytical techniques such as micro-arrays, proteomics, and exciting new tools for the analysis of protein–protein interactions and putative functional protein networks. Some of these studies are on-going in our own laboratory and those of others. As it is likely that most heterogeneous and diverse cancers share a common and obligate mechanistic ancestry—deregulated proliferation and reduced cell death—targeting these pathways should lead to the collapse of the cancer platform. Therefore, the nature of the antiapoptotic mechanism would need to be deciphered in order to reinstate the apoptotic pathway. It seems increasingly likely that future cancer therapies will comprise a combination of specific drug targets as well as chemotherapy and/or radiotherapy. We can optimistically look forward to a future in which the mechanics of c-Myc-induced phenotypes have been elucidated and specific druggable targets in those processes identified.

5.1. Gene Therapy and c-Myc

Recent progress has led to the development of genetic therapy, which can be grouped under two general headings: gene therapy and antisense therapy. The second is to deliver to the target cells antisense oligodeoxynucleotides (ODNs) that can hybridize with mRNA and specifically inhibit the expression of pathogenic genes. With encouraging results from preclinical and clinical studies of ODNs in the past decade, significant progress has been made in developing antisense therapy, with the first antisense drug now being approved for clinical use. To date, phase I and II clinical trials have only been reported for Ras, Bcl-2, and H-Ras and for c-Myc only in the context of coronary heart disease interventions. For recent reviews of antisense approaches, the author recommends Wang et al. (205).

5.2. c-Myc Antisense Oligodeoxynucleotides and Cancer

Many studies have now confirmed the utility of using ODNs to target *c-Myc* expression in cultured cells. Estrogen induces a fivefold increase in c-Myc protein expression within 90 min in steroid-deprived MCF-7 breast cancer cells. Exposure of MCF-7 breast cancer cells to a c-Myc ODN demonstrated up to 95% inhibition of c-Myc protein expression and inhibited estrogen-stimulated cell growth by up to 75% (206).

Similar promising results have now also been shown in animal tumor models both as monotherapy and in combination with traditional chemotherapeutic agents. Continuous subcutaneous perfusion of ODNs to N-c-Myc in human neuroectodermal tumors grown in athymic mice *in vivo* led to a mean 50% reduction in tumor mass (207).

Because many chemotherapeutic drugs kill tumor cells by inducing apoptosis, cellular resistance to apoptosis is thought to be a major factor limiting drug efficacy. Elucidation of those factors dictating whether a tumor will be sensitive or resistant to chemotherapy-induced apoptosis is a major goal in developing future new therapies for individual cancer patients. Chemotherapy resistance in lung cancer therapy has been found to correlate with amplification and overexpression of *c-Myc*. Using a Lewis lung syngeneic drug-resistant murine tumor model Knapp et al. (208) have shown that inhibition of *c-Myc*, using Morpholino oligomers (novel, stable, ODNs for inhibition of gene expression by an RNase H-independent mechanism), to mice previously given cisplatin led to a dramatic decrease in tumor growth rate. Interestingly using both agents concurrently was no more effective than using a single agent only, suggesting that timing of treatments may also be an important factor.

5.3. *c-Myc* Antisense Oligodeoxynucleotides and Coronary Artery Restenosis

Restenosis, resulting from neointimal hyperplasia, is the most important long-term limitation of stent implantation for coronary artery disease, occurring in 15 to 60% of patients. Over 1,500,000 percutaneous coronary interventions are performed annually. Growth and migration of vascular smooth-muscle cells result in neointimal proliferation after vascular injury and are the key mechanism of in-stent restenosis. The rationale of recent approaches to restenosis (e.g., brachytherapy and immunosuppressive agents) is based on the perceived similarities between tumor-cell growth and the benign proliferation of intimal hyperplasia. Several immunosuppressants have been tested for their potential to inhibit restenosis, with the novel strategy of administering the drug via a coated stent platform. Local drug delivery achieves higher tissue concentrations of drug without systemic effects, at a precise site and time. The first multicentre trials were with stents coated with sirolimus and there have now been several other studies showing the efficacy of sirolimus and also paclitaxel (209). Given the known role of *c-Myc* in the proliferation of vascular smooth muscle cells, studies in animal models were undertaken and showed that restenosis after arterial injury or intervention might be profoundly reduced by administering antisense ODN directed against *c-Myc*. Intramural delivery of an advanced *c-Myc* neutrally charged antisense morpholino compound completely inhibited *c-Myc* expression and dramatically reduced neointimal formation in a dose dependent fashion in a porcine coronary stent restenosis model (210). Sadly, a recent trial in patients designed to determine whether this approach could inhibit restenosis when given by local delivery immediately after coronary stent implantation has now been completed (211) and failed to demonstrate significant differences in angiographic restenosis rates.

5.4. Alternative Approaches to Targeting *c-Myc*

Various other approaches have been developed in order to modulate *c-Myc* activity. The application of RNA interference (RNAi) has been explored for some *c-Myc*-target genes in vitro (i.e., for the gene encoding min53; *c-Myc*-induced nuclear antigen with a molecular mass of 53 kDa), with some success (212). In a recent study, it has been shown that *c-Myc* (which bears the potential in its promoter to form a G-quadruplex) can be inhibited by the cationic porphyrin TMPyP4, which is able to bind to and stabilize G-quadruplexes (213).

Indirectly, *c-Myc* may be targeted in several different ways, including through modulation of Max or members of the Mad family, which can regulate transcriptional activity

of c-Myc, or through interference with signaling upstream of c-Myc expression, such as beta catenin. Mutations in the adenomatous polyposis coli (APC) gene, which initiate almost all human colon cancers, directly target the proto-oncogene, *c-Myc*, by elevating β -catenin/T-cell factor (TCF) signaling. Agents that are chemopreventive for colon cancer also stimulate β -catenin/TCF activity in vitro and can result in decreased c-Myc expression (214).

Collier et al. (215) have shown that in rat hepatocytes in vitro the glucose-mediated induction of L-type pyruvate kinase and glucose-6-phosphatase mRNA levels were diminished by recombinant adenoviral vectors that interfere with c-Myc function through a dominant-negative Max protein. Interestingly, recent intriguing data suggest that over-expression of Mad, an antagonist of c-Myc transcriptional activity, may enhance some features of β -cell differentiation, including insulin secretion. Therefore, a future alternative approach to *c-Myc* antisense may be through the targeting of either the c-Myc partner protein Max or antagonist Mad.

6. Future Directions

It is clear that c-Myc is an attractive treatment target in cancer and other diseases of hyperproliferation and also in diabetes. Compelling results from rodent models and cell-culture experiments suggest that future developments in c-Myc-targeted therapeutics hold great promise. However, with the important caveat that successful results in treating human diseases have yet to be demonstrated.

Intriguing paradoxes have been revealed by recent studies; for example, the demonstration that transient inactivation of c-Myc may be sufficient to arrest and reverse certain tumor models but not others, that in some cases seemingly complete regression is followed at some later stage by recrudescence from dormant cells that have escaped apoptosis, and finally that some cancer cells lose their dependence on c-Myc and fail to regress currently seem to complicate analysis, but on the other hand these features suggest that animal models may more completely model human cancer than has previously been appreciated.

Cells in metazoans must compete with one another for growth and survival signals. Intriguing studies with the *Drosophila* homolog of Myc (dMyc) activity have now shown that Myc levels may influence the ability of cells to compete. Cell competition may play a key role during *D. melanogaster* development. Recent studies have suggested that cells expressing higher levels of Myc than their neighbors may actually induce apoptosis in those cells with the lower levels of Myc- in other words such cells act as “super competitors” (126–128). It is not known whether such a phenomenon also exists in vertebrates.

Another criticism that has been levied against mouse cancer models relates to the perceived numerical differences in genetic lesions required for cancer to develop (223). Thus, immortalization of human fibroblasts required perturbation of six pathways-involving p53, pRb, PP2A, telomerase, Raf, and Ral-GEFs. Epithelial cells may require inactivation of the Rb/p16(INK4a) pathway and expression of the catalytic subunit of telomerase, hTERT, in combination with viral oncoproteins. However, it is possible that there are differences in the mutational requirements for “immortalization” and “transformation” of a cell in culture as compared to formation of a cancer cell in vivo. Moreover, it is also possible that some oncogenes, such as c-Myc, may take the place of multiple

other potential lesions because of their diverse roles in promoting numerous cancer behaviors. It is interesting with this in mind that a very recent study has shown that c-Myc may be sufficient to transform a human prostate epithelial cell (224); stabilizing telomere length through up-regulation of hTERT expression and overriding the accumulation of cell cycle inhibitors, such as p16(INK4a). However, these transformed cells still retained some features of normal cells.

Ultimately, we will no doubt resolve these issues, but it seems likely that simply targeting c-Myc or its downstream mediators may only be part of the story and that timing and dosing strategies for such treatments, both alone and in combinations with other cancer treatments will likely prove equally important.

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Role of Lysophospholipids in Cell Growth and Survival

Xianjun Fang and Sarah Spiegel

Summary

Lysophospholipids are not only metabolites in membrane phospholipid synthesis, but also extra-cellular bioactive mediators of multiple biological processes. The best characterized of these are lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P), which have both emerged as important regulators of cell growth and survival. Identification of LPA and S1P receptors in the past several years has led to the accumulation of evidence that most cellular responses to LPA and S1P are mediated through activation of their cognate G protein-coupled receptors (GPCR). There are at least three high-affinity receptors for LPA—named LPA₁, LPA₂ and LPA₃—and five receptor subtypes for S1P, designated S1P₁₋₅. The widespread expression of these receptors and their downstream G proteins of various classes allow activation of a variety of signal transduction pathways that are integrated to trigger diverse cellular responses to LPA or S1P.

This chapter reviews and puts into context current understandings of LPA and S1P receptors and their downstream signaling pathways involved in proliferative and survival responses to LPA and S1P. The significance of these biological responses in human health and disease, particularly potential roles of these lipid mediators in human carcinogenesis, is also discussed.

Key Words: Lysophosphatidic acid; sphingosine-1-phosphate; signaling; metabolism; apoptosis; cell growth and survival; cancer.

1. Lysophospholipid Metabolism

1.1. Lysophosphatidic Acid (see Fig. 1)

Although LPA is considered to be a key intermediate in *de novo* lipid synthesis in all cells formed by acylation of glycerol 3-phosphate catalyzed by glycerophosphate acyl-transferase (1), little is known of how its extracellular levels are regulated. LPA can be produced by activated platelets, adipocytes, fibroblasts, endothelial cells, and ovarian and prostate cancer cells from newly generated phosphatidic acid by the action of phospholipase D followed by phospholipase A1 or A2-mediated deacylation (2–6). It was recently suggested, however, that the bulk of LPA arising from platelet activation results from the sequential cleavage of serum and membrane phospholipids to lysophospholipids by PLA1 and PLA2 secreted by platelets, followed by conversion to LPA by lysophospholipase D (LysoPLD) activity present in plasma (7) (see Fig. 2).

Plasma LysoPLD was recently purified and identified as autotaxin (ATX/NPP-2), a member of the nucleotide pyrophosphatase and phosphodiesterase family of exo- and ecto-enzymes (8,9). ATX was originally identified as a tumor cell motility-stimulating factor (10). Intriguingly, stimulation of tumor cell migration and invasion is also one of

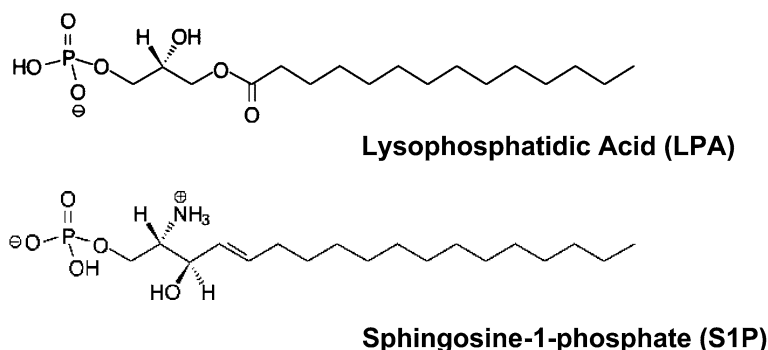


Fig. 1. Structures of LPA and S1P.

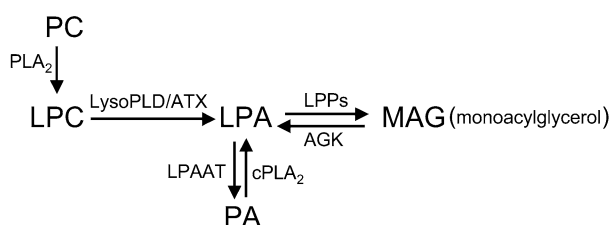


Fig. 2. Major pathways for biosynthesis and metabolism of LPA.

the major biological functions of LPA ([11](#)), suggesting that ATX promotes cell motility via formation and action of LPA.

Another potential pathway for synthesis of LPA is phosphorylation of monoacylglycerol by a specific lipid kinase activity first described more than 30 yr ago which has received scant attention following the characterization of the partially purified bovine brain enzyme in 1989 ([12](#)). Recently, a novel lipid kinase that phosphorylates monoacylglycerol to form LPA, named AGK, has been identified ([13](#)).

LPA can be acylated at the *sn*-2 hydroxyl group by acyl transferases, such as endophilin, to produce phosphatidic acid, another important lipid mediator ([14](#)), deacylated by lysophospholipases to glycerol phosphate ([15](#)), or dephosphorylated by lipid phosphate phosphohydrolases (LPPs), including LPP1/PAP2A, LPP3/PAP2B and LPP2/PAP2C, to generate monoacylglycerol ([16–19](#)). LPPs are located at the cell membrane with their catalytic domains facing the extracellular media and degrade LPA associated with the external leaflet of the membrane. Overexpression of LPPs has been shown to antagonize cellular responses to LPA and reduce LPA concentrations in the medium ([18,19](#)). In contrast, inhibition of platelet LPP sensitizes their responses to LPA and also increases thrombin-induced LPA production ([20](#)). Overall, these data suggest that LPPs play a pivotal role in controlling extracellular levels of LPA and its functions.

1.2. Sphingosine-1-Phosphate (see [Fig. 1](#))

Sphingosine, the backbone of all sphingolipids, is converted to S1P intracellularly by sphingosine kinase (SphK)-mediated phosphorylation ([21](#)). Sphingosine is not an intermediate in the biosynthesis of sphingolipids, but rather is produced by ceramidase

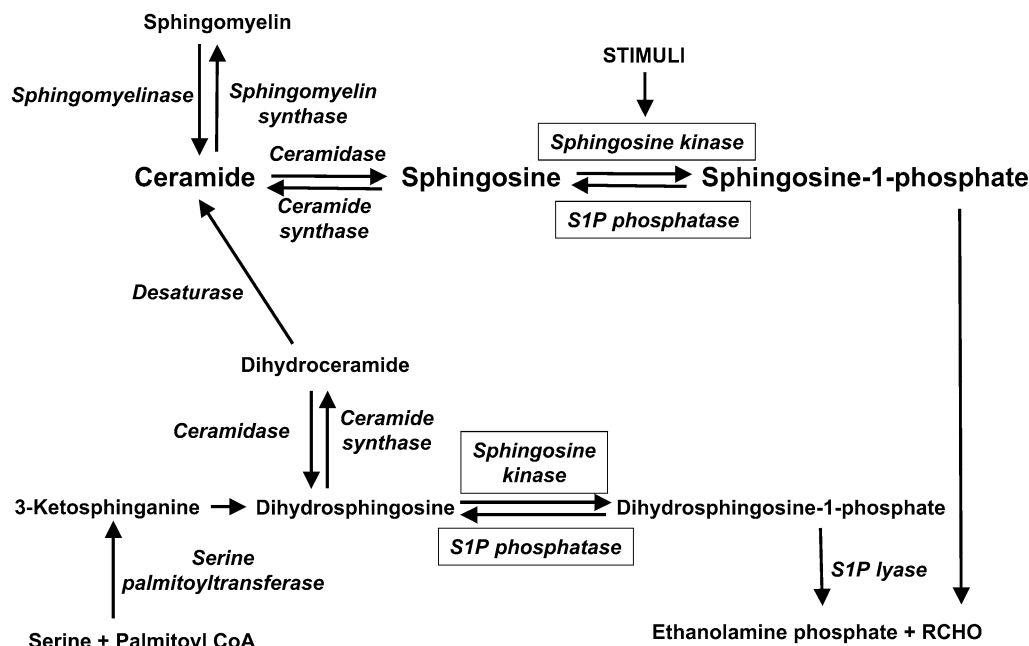


Fig. 3. Biosynthesis of sphingolipids and metabolism of S1P.

catalyzed degradation of ceramide, a fatty acid amide linked sphingoid base, of which sphingosine is the most predominant in mammalian cells. Ceramide is generated by a *de novo* biosynthetic pathway in which the intermediate sphinganine (dihydrosphingosine) is *N*-acylated to dihydroceramide prior to introduction of the 4–5 *trans* double bond characteristic of sphingosine (22) (see Fig. 3). Several different types of sphingomyelinases, which are activated by diverse cellular stresses, degrade membrane sphingomyelin to also produce ceramide. Intracellular levels of these interconvertible sphingolipid metabolites, ceramide, sphingosine, and S1P, are typically low and are determined by the balance between synthesis and degradation.

S1P can be reversibly degraded to sphingosine by dephosphorylation catalyzed by S1P phosphohydrolases. Two S1P-specific phosphohydrolases, SPP-1 and SPP-2, have been cloned (23,24) and both are located in the endoplasmic reticulum, suggesting that they are involved in dephosphorylation of intracellular S1P. An alternative degradation pathway is mediated by S1P lyase, a pyridoxal phosphate-dependent enzyme that cleaves S1P irreversibly to phosphoethanolamine and hexadecenal (25).

Two isoforms of SphK (SphK1 and SphK2) are expressed in mammalian tissues and cells (26,27). Database searches have identified homologous SphK genes in plants, insects, worms, and zebra fish, indicating that the S1P-generating mechanism is conserved throughout evolution (21). There is a rapidly growing list of agonists that have been reported to increase SphK activity, including growth factor receptor tyrosine kinases (28,29), ligands for GPCRs (30,31), cross-linking of immunoglobulin receptors (32–35), the protein kinase C (PKC) activator phorbol ester (36), and inflammatory cytokines, such as tumor necrosis factor (TNF)- α (37). So far, no agonists have been described that affect SphK2 activity. Although SphK2 is highly similar to SphK1 and has the same five conserved domains, it diverges in its central and N-terminal regions,

and has a different developmental and tissue expression pattern (27), suggesting that these two isoenzymes have distinct physiological functions. Indeed, whereas SphK1 promotes growth and survival (38), SphK2 suppresses growth and enhances apoptosis (39,40). It is still unclear whether SphK2 has redundant or antagonistic physiological functions to SphK1.

The mechanisms by which extracellular stimuli activate SphK1 and S1P production are beginning to be unraveled. A number of studies have addressed the involvement of intracellular calcium signaling in agonist-induced SphK1 activation (31,41–43). In PDGF-stimulated cells, the phospholipase C (PLC) signaling pathway and calcium mobilization is required for SphK1 activation (41). Consistent with the importance of calcium mobilization, SphK1 contains putative calcium/calmodulin binding domains (26). A clue to the mechanism of SphK1 activation comes from the observation that the majority of SphK1 is found in the cytosol, whereas the hydrophobic substrate sphingosine is generated in membranes (38,44). Growth factors and FcεRI crosslinking induce translocation of SphK1 to plasma membrane regions in various types of cells (35, 45–47). This translocation could increase SphK1 activity, either through direct association with an activating membrane component, such as acidic phospholipids (48), or by bringing it in close proximity to its substrate sphingosine. Although activation of PKC by phorbol ester (47) and vascular endothelial growth factor (VEGF) (49) induces phosphorylation of SphK1 and translocation to the plasma membrane, PKC cannot phosphorylate SphK1 directly, suggesting the participation of other protein kinases. In agreement, phosphorylation of overexpressed SphK1 on serine 225 by ERK2 induced by TNF-α not only increased its activity but was also necessary for its translocation from the cytosol to the plasma membrane (50). Although a phosphorylation deficient SphK1 mutant retained full catalytic activity, it lost its ability to induce proliferation, form colonies in soft agar, and induce tumor formation in nude mice (51). These results suggest that phosphorylation of SphK1 is essential for oncogenic signaling and is brought about by phosphorylation-induced translocation to the plasma membrane rather than enhanced catalytic activity. Another report suggested that SphK1 is translocated to membrane compartments enriched in phosphatidic acid (PA), produced by PLD. This translocation likely involves direct interaction of SphK1 with PA, as the purified enzyme has been shown to bind tightly to immobilized PA (52).

An unresolved issue in S1P signaling is how S1P exits cells to serve as a ligand for multiple cell surface S1P receptors. The bulk of extracellular S1P seems to be secreted by unidentified trafficking mechanisms (7). It remains unknown whether S1P is also generated extracellularly, for example, by secreted SphK1 (53). In addition, ATX, the enzyme that produces extracellular LPA (8,9), has been reported to be able to hydrolyze sphingosylphosphorylcholine (SPC) to S1P, although the catalytic activity towards SPC is much weaker than with lysophosphatidylcholine (LPC) (54).

2. Signal Transduction of LPA and S1P

Research and interest in lysophospholipid mediators was greatly accelerated by the discovery of their specific cell surface receptors. Since the cloning of the first LPA receptor in 1996 (55), sequence similarities have allowed rapid identification of multiple S1P receptors and additional LPA receptor subtypes. The four LPA receptors identified to date are LPA₁, LPA₂, LPA₃ (56), and the newly identified LPA₄/GPR23

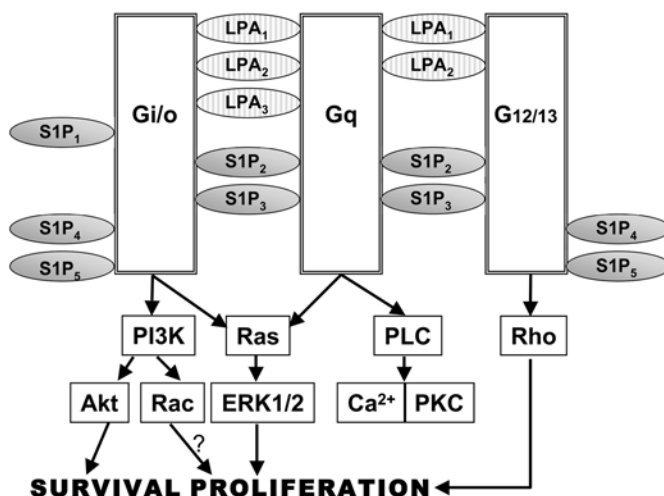


Fig. 4. LPA and S1P signaling important for cell growth and survival. A simplified scheme illustrating coupling of LPA and S1P receptors with different classes of G proteins, leading to activation or inhibition of downstream signaling involved in cell growth and survival.

receptor (57), which is structurally distinct from other three LPA receptors. Whether LPA₄/GPR23 is a bona fide LPA receptor is still controversial. Five cognate GPCRs for S1P (S1P₁₋₅) have been identified and their signaling analyzed in detail (58,59). It has recently been claimed that the orphan receptors GPR3, GPR6, GPR12 are authentic S1P receptors (60), but this assignment needs further confirmation.

Whereas some other types of GPCRs show selectivity in interactions with particular G proteins, most of the LPA and S1P receptor subtypes appear to couple to multiple heterotrimeric G proteins (*see* Fig. 4). LPA₁, LPA₂, S1P₂, and S1P₃ couple to Gi, Gq, and G12/13 (61). LPA₃ interacts with Gi and Gq but not G12/13 (61). S1P₁ interacts exclusively with Gi, whereas S1P₄ and S1P₅ are capable of coupling to both Gi and G12/13 (61).

LPA- or S1P-induced activation of Gi, which is sensitive to pertussis toxin, leads to inhibition of adenylyl cyclase (AC) and decreased cAMP, as well as activation of the Ras/MAPK cascade and the PI3 kinase-Akt pathway mediated by $\beta\gamma$ subunits released from Gi (61–63). Decreased cAMP favors cell growth in many types of cells (64,65). Thus, Gi-dependent inhibition of AC may contribute to proliferative responses to LPA and S1P. The MAPK and PI3K pathways downstream of the $\beta\gamma$ dimer are integral components of the proliferative and survival signaling networks as discussed below.

Gq binds to and activates PLC β , which, in turn, catalyzes hydrolysis of phosphatidylinositol bisphosphate (PIP₂) to diacylglycerol (DAG) and inositol trisphosphate (IP₃) (66). DAG activates PKCs and IP₃ triggers intracellular calcium mobilization. Although activation of some Gq-coupled receptors or overexpression of constitutively active Gq can result in cell proliferation, activation of PLC β , Ca²⁺ and PKC signaling pathways are generally insufficient to promote cell proliferation or survival (63).

LPA- or S1P-induced activation of G12/13, which directly binds to the Rho-specific guanine nucleotide exchange factor p115RhoGEF, leads to Rho activation (67,68). Rho signaling was once considered to be solely involved in remodeling of the actin

cytoskeleton, ultimately controlling cell morphology and a variety of functions, such as cell motility, aggregation, polarity, and contraction (69). It has become evident that Rho also regulates expression of genes associated with cell proliferation including c-jun, c-fos, and cyclin A (70). S1P induces association of the scaffold protein CNK1 with p115RhoGEF, stimulating the JNK/MAPK cascade and thus controlling signaling specificity downstream of Rho (71). A critical role for Rho in cell-cycle progression is further suggested by its ability to antagonize Ras and suppress expression of the cell-cycle inhibitors p21 and p27 (72,73), thus facilitating entry into the S phase of the cell cycle.

The signaling pathways and ultimate biological consequences vary from one cell type to another depending on the spectrum and expression levels of the receptors and the specific G proteins present in the cell and possible “cross-talk” with signals mediated by other families of cell-surface receptors. In many cell types, treatment with either LPA or S1P can activate growth factor tyrosine kinase (TK) receptors, such as endothelial growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR), and c-met (the receptor for hepatocyte growth factor), in the absence of added growth factors, indicating that receptor TKs might be activated through intracellular receptor crosstalk (also known as transactivation) (74–76). This cross-communication between different signaling systems is not only important for the growth promoting activity of LPA (74), it also may be a clue to its pathophysiological role in prostate cancer (75), head and neck squamous cell carcinoma (77), and kidney and bladder cancers (78).

Moreover, activation of receptor TKs or cross-linking of FcεRI can induce a reverse type of transactivation of S1P receptors that may be important for effects on cell movement (35,45,79). According to this paradigm, stimulation of PDGFR activates and translocates SphK1 to the plasma membrane. This results in spatially restricted formation of S1P, that, in turn, activates S1P₁, resulting in activation and integration of downstream signals, such as the small GTPase Rac, important for protrusion of lamellipodia and forward movement (45,79). However, ligation by S1P will suppress migration towards PDGF when S1P₂ is highly expressed (80). These results revealed a role for receptor cross-communication in which activation of S1PRs by a receptor tyrosine kinase is critical for cell motility and shed light on their vital roles in vascular maturation (81) and angiogenesis (82,83). Moreover, transactivation of S1PRs by FcεRI triggering is important for normal mast cell degranulation and chemotaxis towards antigen and might be involved in the movement of mast cells to sites of inflammation (35). In addition, differential transactivation of S1PRs by nerve growth factor (NGF) regulates antagonistic signaling pathways that modulate neurite extension and might be important during development and differentiation of the central nervous system (84).

3. LPA and S1P as Growth Factors

Most of the initial studies of biological effects mediated by exogenous LPA and S1P have centered on their mitogenic activity in fibroblasts. Both LPA and S1P, when exogenously applied to quiescent fibroblasts, stimulate cellular DNA synthesis and cell division (85,86). Other related phospholipids, such as PA, LPC, sphingosine, and SPC have been also reported to have variable mitogenic effects on fibroblasts or other cell types (85,87,88). Later studies suggested that the mitogenic activity of these LPA- and

S1P-related phospholipids most likely results from the contamination of the lipid preparations with LPA or S1P or conversion into LPA or S1P.

A common feature of LPA- and S1P-induced mitogenesis is the requirement of relatively high doses (μM) of these ligands for significant mitogenic activity (85,86,88). This requirement differentiates LPA and S1P from peptide growth factors such as EGF or PDGF, where nanomolar concentrations are usually sufficient to trigger pronounced DNA synthesis (85,89). Because of the potential membrane lytic activity of high concentrations of LPA and S1P, it was initially argued that they might trigger cellular DNA synthesis through membrane perturbations. It is now clear that LPA is quickly degraded in culture by membrane-associated LPPs, thus limiting its functional concentrations at cell membranes (16–19,90). A relatively high initial concentration of LPA appears to be critical for maintaining a threshold level of the ligand to elicit DNA synthesis. Recent studies from several groups demonstrated that the dose of LPA required for its functions could be markedly reduced by modifying its structure (20,91). For example, replacement of the phosphate group of LPA with a phosphothionate has produced LPP-resistant LPA analogs with 10- to 100-fold increases in their potency to stimulate cell proliferation (91).

Mitogenic effects of exogenous LPA and S1P have been observed in a wide range of cell types, including epithelial cells, endothelial cells, smooth muscle cells, astrocytes, renal mesangial cells, and various transformed cells (62,63). Although LPA and S1P increase IP_3 formation and mobilize intracellular calcium in these cells, PKC activity or calcium signaling seems neither necessary nor sufficient for LPA- or S1P-induced cell proliferation (63). As demonstrated in numerous systems, mitogenic responses to LPA or S1P are sensitive to pertussis toxin (85,88,92,93), implicating Gi/o in transduction of signals from receptor activation to cell proliferation. An early study suggested that Gi -induced inhibition of AC and reduction of intracellular cAMP can mediate LPA-stimulated proliferation of Rat1 cells (85). However, a general role for Gi inhibition of cAMP in LPA- or S1P-triggered cell proliferation seems unlikely because LPA still acts as a potent mitogen in some cell types, such as Swiss 3T3 cells and epithelial cells, where cAMP is a positive regulator of cell growth (94,95).

Characterization of other signaling pathways downstream of Gi has revealed the importance of the Ras/ERK and PI3K/Akt pathways in LPA- and S1P-induced cell growth (93,96–98). The universal requirement for ERK activation in cell proliferation likely reflects the necessity for the translocation of activated ERK to the nucleus where it enhances transcription of proliferation-associated genes. Of note, LPA induces biphasic activation of ERK in Rat1 cells, with a transient initial signal and subsequent sustained activation (99). The sustained phase of ERK activation, which is sensitive to PTX, correlates well with the nuclear translocation of ERK and the initiation of cell proliferation.

The mechanism of the dependence on PI3K of LPA or S1P-induced cell proliferation is not fully understood. The most prominent downstream effector of PI3K is the serine/threonine kinase Akt, a key player in cell survival. Akt may contribute to cell growth by preventing apoptotic cell death or may directly modulate the mitogenic process. The lipid products of PI3K also activate other signaling molecules in addition to Akt. It was recently demonstrated that Tiam, a Rac-specific guanine nucleotide exchange factor (GEF), lies downstream of PI3K (100,101). Besides its prominent role

in lamellipodia protrusion and forward cell movement, Rac may also contribute to cell-cycle progression through its effector p21-activated kinase (PAK), superoxide production, and cyclin D1 expression (102–104).

Which receptor subtypes are most intimately involved in LPA- or S1P-induced cell proliferation has not been fully established. Most cell types express more than one LPA or S1P receptor and LPA and S1P generally stimulate proliferation, making it difficult to link a proliferative response to a specific receptor. In theory, the receptor subtypes that couple to Gi and/or G12/13 may be capable of mediating growth responses to a certain degree. Consistent with its ability to activate Gi and G12/13, ectopic expression of the LPA₁ receptor in B103 neuronal cells sensitized LPA-induced DNA synthesis (105). Similarly, the S1P₂ and S1P₃ receptors couple to both Gi and G12/13 and have been shown to mediate growth responses to S1P in hepatoma cells (106). However, other LPA and S1P receptors have also been implicated in cell proliferation (107–109).

It should be emphasized that studies of heterologous overexpression only indicate that a specific receptor has the ability to mediate growth responses but do not necessarily implicate them as physiological regulators. The most meaningful observations concerning the relevance of endogenous LPA receptors in cell proliferation were made recently with fibroblasts (MEFs) derived from specific LPA receptor-null mice (109). The mitogenic response to LPA was only partially compromised in MEFs lacking either LPA₁ or LPA₂ (109). However, LPA was not able to stimulate DNA synthesis in MEFs from LPA₁/LPA₂ double knockouts (109), suggesting that both receptors contribute to the mitogenic effects of LPA. Similarly, the mitogenic effect of exogenous S1P was absent in MEFs devoid of functional S1PRs (110). In normal physiological circumstances, the optimal response to LPA and S1P is likely determined by the relative expression of the endogenous receptors.

Interestingly, both LPA and S1P have been reported to be growth-inhibitory for certain cell types. For example, LPA inhibits growth of myeloma cells (111). This antimitogenic action was proposed to be regulated by LPA-induced increases in intracellular cAMP, an antimitogenic signal for these cells. Further, both LPA and S1P inhibit proliferation of cells of epidermal origin. LPA treatment induced growth arrest of keratinocytes, probably acting through a Smad3-dependent mechanism, similar to the signaling pathways utilized by transforming growth factor (TGF)- β (112). Recent studies have shown that S1P induces rapid phosphorylation of Smad3 and its association with Smad4 leading to translocation of the complex into the nucleus and inhibition of keratinocyte growth (112). Both S1PRs and TGF β 1R were essential, indicating a novel type of cross-talk between serine/threonine kinase receptors and S1PRs. Others have suggested however that the antimitogenic action of S1P in these cells is linked to the inhibition of cyclin D2 expression and associated induction of cyclin-dependent kinase inhibitors p21 and p27 (113). Growth inhibitory activity of S1P was also observed in hepatic myofibroblasts (92), rat hepatocytes (114), and T-lymphocytes (115), but the mechanisms involved have not been delineated.

4. LPA and S1P as Survival Factors

Like peptide growth factors, LPA and S1P also act as dual mediators of cell growth and survival. Frankel and Mills first recognized the survival-promoting activity of LPA based on their observation that LPA protected cisplatin-treated ovarian cancer cells

from apoptotic cell death (116). Numerous studies have since been published that confirm the antiapoptotic activity of LPA in normal and transformed cells of diverse origins, including fibroblasts (93), intestinal epithelial cells (117), Schwann cells (118,119), macrophages (120), T-lymphocytes (121), cardiac myocytes (122), osteoblasts (123), renal proximal tubular cells (124), prostate cancer cells (125), and primary chronic lymphocytic leukemia cells (126). LPA also seems to be effective in preventing apoptosis induced by a wide variety of death inducing agents, including chemotherapeutic drugs (116,117), radiation (117), serum-deprivation (93,124), detachment (anoikis) (4), *Fas* ligand (121,127), and activation of the death receptor TNF-related apoptosis-inducing ligand (TRAIL) (128).

LPA administration also has cytoprotective effects *in vivo*. A study using a renal ischemia reperfusion injury model demonstrated that LPA inhibited apoptosis of tubular epithelial cells in a dose-dependent manner (129). In irradiated mice, oral administration of LPA was shown to decrease the number of apoptotic cells found in intestinal crypts (117). Moreover, rectally applied LPA in rats stimulates intestinal epithelial wound healing and diminishes mucosal damage (130).

However, the underlying mechanisms responsible for this general protective action of LPA manifested *in vitro* and *in vivo* have not yet been fully defined. Some of these observations are consistent with a role for Gi-elicited signals in the antiapoptotic responses to LPA. PI3K, the most prominent survival regulator downstream of Gi, was reported to be required for LPA-dependent cell survival of Schwann cells (118,119), macrophages (120), prostate cancer cells (125), and primary chronic lymphocytic leukemia cells (126). Other studies showed synergism between the PI3K pathway and the Gi-linked ERK1/2 pathway (119,131). In murine fibroblasts, however, Gi-mediated ERK1/2 activation plays a dominant role in LPA survival activity, whereas inhibition of PI3K has only minor effects (93). In addition, in Schwann cells, G12/13-mediated activation of Rho GTPase may also participate in the cytoprotective effects of LPA (132).

Other documented antiapoptotic processes mediated by LPA include suppression of proapoptotic proteins Bax in T-cells (121) and Bcl-2 in rat intestinal epithelial cells (133), inhibition of caspase activity in ovarian cancer cells (133,134), and prevention of cytochrome C release in intestinal epithelial cells (128). Notably, in many cell types, LPA potently induces activation of NF- κ B and NF- κ B-mediated transcription (107,125,135–137). In light of the generally accepted prosurvival role for NF- κ B, its activation by LPA may represent another signaling event related to the survival effect of LPA.

Interestingly, there are reports that in some types of cells, exogenous LPA treatment can promote apoptosis. For example, LPA enhances PMA-induced apoptosis of erythroblastic TF-1 cells by interfering with their adhesion (138). In other cell types such as cortical neurons and airway smooth muscle cells (139), low concentrations of LPA signal survival and/or mitogenesis whereas high levels of LPA lead to apoptosis.

It is now well accepted that S1P enhances survival and protects cells against ceramide-mediated cell death (140–142). This antiapoptotic activity was dependent on activation of ERK1/2. S1P-mediated protection against apoptosis, like that of LPA, appears to be conferred primarily through Gi signaling cascades downstream of S1PRs. In a number of cell types, Gi-mediated activation of the PI3K-Akt cascade plays a primary role in the protective action of S1P (123,128,143). In endothelial cells and

melanoma cells, PI3K-Akt has been further linked to the regulation of expression or functional status of Bcl-2 family members such as Bax, Bcl-2, Bad, and Bim (*144,145*). Gi-mediated activation of the ERK/MAPK pathway has been also found to be instrumental to S1P-enhanced cell survival (*106*). For example, activation of ERK1/2 and concomitant suppression of JNK are required for S1P-mediated protection of murine fibroblasts from ceramide-induced apoptosis (*38,140,142*). Similarly, activation of ERK1/2 by S1P protects melanocytes from UVB-mediated apoptosis (*146*). Like LPA, S1P activates NF- κ B (*147*), which may also be a contributing factor to the cytoprotective effects of S1P.

5. S1P as an Intracellular Mediator of Growth and Survival

As discussed earlier, S1P is mainly generated intracellularly via the action of SphKs. In addition to the well-characterized receptor-dependent functionalities of S1P, several lines of evidence indicate that intracellularly produced S1P can also act within the cell, independent of S1P receptors. The biological effects of S1P, particularly its growth and survival-promoting activity, can be recapitulated by overexpression of SphK1 and intracellular S1P accumulation in the absence of detectable cell surface S1PRs or when specific S1PRs or downstream signaling are pharmacologically inhibited (*110*). However, in the same cell, migratory responses to growth factors were dependent on the presence and function of S1P receptors, indicating pleiotropic cellular effects of S1P are mediated through both intracellular and extracellular routes (*110*). Consistent with this postulated intracellular action, S1P was initially recognized as an intracellular regulator of cell growth. Furthermore, stimulation of mammalian cells with many growth factors, such as PDGF or VEGF, triggers activation of SphK1 and the subsequent accumulation of intracellular S1P. Substantial evidence exists that the intracellular production of S1P is essential for PDGF-induced cell proliferation and survival (*28,41,110,148*). It was recently found that VEGF can also stimulate SphK1 and this has ramifications for its mitogenicity (*49*). VEGF stimulation of PKC and consequent activation of SphK1 resulted in the conversion of sphingosine to S1P, and concomitant inhibition of Ras GTPase-activating proteins (*149*), leading to activation of the ERK21/2 pathway and cell proliferation (*49*). Several other lines of evidence further support the notion that S1P produced in response to growth factors also has an intracellular action independent of S1PR engagement: (1) dihydro-S1P, which is identical to S1P and only lacks the 4,5-*trans* double bond, binds to all of the S1PRs and activates them, yet does not mimic the effects of S1P on cell survival (*37,45,141,150*); (2) microinjection of S1P, which elevates intracellular S1P, has been shown to mobilize calcium (*42*) and enhance proliferation and survival (*141,150,151*); (3) SphK1 mediates VEGF-induced activation of Ras without involving S1PRs (*49*); (4) yeast and plants do not possess S1PRs, yet phosphorylated long-chain sphingoid bases regulate environmental stress responses (*152,153*) and other important biological responses (*154,155*). In contrast to the well-established receptor-mediated processes, the mechanisms for these intracellular actions of S1P are poorly understood. Identification of the protein targets or downstream effectors of S1P within the cell will provide insights into the mechanistic steps leading to S1P-induced cell proliferation and survival.

Whether LPA similar to S1P also has intracellular actions is not well established, as substantial evidence indicates that LPA acts essentially from outside of the cell through

its cognate receptors and microinjection of LPA does not elicit similar cellular responses (156). However, the mitogenic responses of some mammalian cells to LPA may be LPA₁₋₄ independent (17). It has been suggested that LPA has a novel intracellular function as a high-affinity ligand for peroxisome proliferator-activated receptor (PPAR) γ (157), a transcription factor that regulates genes controlling energy metabolism (158) and can exacerbate mammary gland tumor development (159). Indeed, LPA induced proliferation of mast cells was attenuated by GW9662, a selective antagonist of PPAR γ (160). Moreover, LPA activates PPAR γ in yeast which do not express LPARs (158).

6. LPA and S1P in Health and Disease

Both LPA and S1P are secreted by activated platelets and hematopoietic cells at the platelet-endothelial cell interface. In humans, serum LPA levels increase within 1 to 24 h of blood clotting from approx 1 to 5–6 μM (161). S1P is abundantly stored within platelets which have high levels of SphK activity and low levels of S1P lyase activity (162,163). S1P concentration in serum ranges from approx 0.5 to 0.8 μM (163). In vivo, LPA is rapidly and locally produced in response to injury, inflammation and stress conditions (1). A number of studies in animals have shown that LPA promotes wound healing and tissue regeneration. For example, topically applied LPA promotes wound healing in skin (164), whereas rectally administered LPA accelerates intestinal wound healing and reduces mucosal damages (130). These protective effects of LPA have been ascribed largely to LPA's growth and survival-promoting activity towards epithelial and mesenchymal cells. In addition, LPA stimulates migration and contraction of these cells (165,166), processes critical for normal wound healing.

Characterization of S1P₁ knockout mice has revealed a crucial role for S1P in angiogenesis and vascular maturation during development. The S1P₁ receptor null mice exhibit embryonic lethality at embryonic day (E)12.5–E14.5 resulting from defects in vascular smooth muscle migration (81). Consistent with the phenotypes of the S1P₁ knockout mice, S1P stimulates proliferation, migration and differentiation of endothelial and smooth muscle cells in culture. In an in vitro wound healing assay, S1P stimulated closure of wounded monolayers of human umbilical vein endothelial cells and adult bovine aortic endothelial cells (167). Thus S1P may contribute to vascular remodeling during tissue regeneration.

Both LPA and S1P have been implicated in the pathogenesis of malignant diseases. They potentially participate in multiple processes associated with cancer development and metastatic progression, including cell proliferation, evasion of apoptosis and chemotherapeutic killing, and tumor-cell migration and invasion. Accumulated evidence supports the notion that in many types of cancers, LPA or S1P production is deregulated and/or the expression of their corresponding receptors is upregulated, leading to aberrant signaling. In this regard, the role of LPA in ovarian cancer has been studied most extensively. High concentrations of multiple forms of LPA are found in ascites from ovarian cancer patients (89,168).

As ascites fluid represents the in vivo microenvironment of tumor cells, high levels of LPA could affect the growth, survival, motility, and fate of ovarian tumor cells. Importantly, we have recently found that the LPA₂ and LPA₃ receptors are overexpressed in more than 50% of primary ovarian cancers and in established ovarian cancer cell lines

compared with normal ovarian epithelial cells where only the LPA₁ receptor is present (4,107). LPA potently induces ovarian cancer cell proliferation, survival and expression of oncogenic proteins, including uPA, VEGF, IL-6, and IL-8, whereas these responses in normal ovarian epithelial cells are absent or limited (107,169–171). It is tempting to speculate that the acquired expression of LPA₂ and LPA₃ may be responsible for the differential responses to LPA of normal and malignant ovarian epithelial cells (107,170).

In addition to ovarian cancer, overexpression of specific LPA receptors has been also found in colon cancer and colorectal cancer (172,173), differentiated thyroid cancer (174), and invasive ductal carcinomas (175), indicating that amplification of LPA signaling through overexpression of LPARs may be a common oncogenic process. A recent study demonstrated that LPA₁₋₃ are also expressed by primary breast cancer cells (176). Ectopic overexpression of LPA₁ in MDA-BO2 breast cancer cells resulted in enhanced potential of these cells to metastasize to the bone in nude mice (176). In contrast to ovarian cancer cells that may synthesize LPA, MDA-BO2 cells do not produce or secrete LPA. Instead, LPA was released from platelets that were aggregated and activated around the tumor cells. Therefore, the platelet antagonist Integrilin effectively blocked bone metastasis induced by overexpression of the LPA₁ receptor in MDA-BO2 cells (176). This study implicates LPA as an endogenous factor in the regulation of metastatic progression of breast cancer.

A critical role for S1P signaling in oncogenesis is evidenced by the observation of the oncogenic effect of the S1P-producing enzyme SphK1 (177). NIH 3T3 cells overexpressing SphK1 acquired the transformed phenotype as determined by focus formation, anchorage-independent growth in soft agar, and tumorigenicity in NOD/SCID mice (51,177). Cellular SphK1 activity was found to underlie oncogenic Ras-induced transformation of NIH 3T3 cells (177). We also demonstrated that SphK1 over-expression in estrogen-dependent MCF-7 breast cancer cells led to a more tumorigenic phenotype when implanted in nude mice (178). Moreover, expression of a dominant-negative mutant SphK1 profoundly inhibited estrogen-mediated ERK1/2 activation and neoplastic cell growth (179). These results suggest that SphK1 activation is important in transduction of estrogen-dependent mitogenic and carcinogenic action in human breast cancer cells. The ability of S1P to act in an autocrine or paracrine manner, combined with its actions on angiogenesis and vascular maturation that are also critical for tumor progression, suggest that SphK1 may not only protect tumors from apoptosis and enhance growth, it may also increase their vascularization. Because inhibitors of SphKs, such as *N,N*-dimethylsphingosine (DMS) and *L*-threo-dihydrosphingosine (known as safingol), induce apoptosis regardless of multidrug resistance expression (180), they may provide a new strategy for treatment of anticancer drug-resistant cancers. In pilot clinical phase I trials with safingol, which also potentiated the tumor-inhibiting effect of doxorubicin in tumor-bearing animals, it was found that safingol can be given safely with doxorubicin (181). Indeed, SphK inhibitors reduce gastric tumor growth (182) and mammary adenocarcinoma tumor growth in mice (183). Because SphK1 is overexpressed in a variety of solid tumors, including breast, stomach, ovary, kidney, and lung, compared with normal tissues from the same patients (183), anticancer therapeutics targeting this SphK isozyme might be useful adjuncts for management of many types of cancer.

As discussed above, S1P signaling through the S1P₁ receptor is required for stabilization of nascent blood vessels during embryonic development. Besides its growth- and survival-promoting activity, S1P may mediate tumor angiogenesis which is critical for the growth of tumors beyond a diffusion-limited size and for metastasis (184). A recent study showed that S1P₁ expression is strongly induced in vessels within tumor xenografts (185). Blocking its expression on neovessels with siRNA suppressed tumor angiogenesis and tumor growth in nude mice. These results provide support the involvement of S1P in tumor angiogenesis, further indication that S1P signaling deserves consideration as a therapeutic anticancer target.

As prototypic ligands of GPCRs, LPA and S1P have been the subjects of extensive research. Their potential roles in pathogenesis of human cancer are only beginning to be elucidated. Although the earliest work that implicated LPA and S1P in cancer focused on only a few cancer models, such as ovarian and breast cancer cells, more evidence is accumulating to support a general role for LPA and S1P in the development and progression of human malignancies. This can be explained by the fact that both LPA and S1P are general growth and survival factors with a broad range of target tissues and cell types. The relevance of LPA and S1P to abnormal growth control in cancers is further highlighted by the defects or abnormalities in LPA and S1P metabolism and their receptor expression/functions found in multiple types of human cancers. LPA and S1P (as small lipids) and their GPCRs are highly “drugable” targets. In fact, more than half of all drugs in current use target GPCRs. Future studies will no doubt uncover novel therapies intervening in production and/or receptor signal transduction pathways of LPA and S1P.

Acknowledgments

We apologize to those authors whose work could not be cited owing to space limitations. This work was supported by National Institutes of Health grants CA102196 (XF), GM43880 (SS), CA61774 (SS), and AI50094 (SS), and the Department of Defense grants W81XH-04-1-0103 (XF) and PC10392 (SS). The authors thank Dr. Sheldon Milstien for helpful comments.

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Alternative Use of Signaling by the β GBP Cytokine in Cell Growth and Cancer Control

From Surveillance to Therapy

Livio Mallucci and Valerie Wells

Summary

β GBP is an antiproliferative cytokine produced by activated T-cells and endogenously released by somatic cells. In normal cells, β GBP negatively regulates the cell cycle; in cancer cells β GBP induces apoptosis. Mechanisms of action involve downregulation of PI3 kinase via p110 targeting. Downregulation of PI3 kinase by β GBP reflects on Ras-ERK signaling and Akt mediated signaling resulting in the delay of S/G2 transition in normal cells and activation of programmed cell death in cancer cells. The apoptotic response of cancer cells to β GBP takes place according to the molecular context of the cells. Where β GBP induces cell-cycle arrest, apoptosis relates to E2F-1 deregulation. In cancer cells characterized by strong mitogenic input and elevated Akt/PKB expression, loss of Akt protein deprives the cells of survival signaling. β GBP is an immunomolecule which can perform therapeutically what the native, endogenous protein would naturally perform in a surveillance role.

Key Words: Cancer therapy; apoptosis; E2F-1; PI3 kinase; Akt/PKB; β GBP; β -galactoside binding protein.

1. Signaling and Targeting

Within the context of cell proliferation, differentiation, and apoptosis, the fate of a cell is strictly determined by molecular events where an integrated cascade of signals connects molecules with phenotypes. Positive and negative regulatory cascades control transcriptional events and the dynamics of cell-cycle transitions (1–3). Orderly cell replication depends on the balanced and time-programmed expression of proto-oncogenes, tumor suppressor genes, and associated events. Deregulation of this balance in favor of proliferation is a prime step toward tumorigenesis, where progression to malignancy depends on increased reliance on survival signaling as increasing mitogenic input—a consequence of multiple mutations—requires compensatory suppression of proapoptotic processes in order to allow uncontrolled expansion (4–6). Alterations of the Ras-ERK and the PI3K/Akt pathways are key to successful tumorigenesis (6–8). Knowledge of the integrated system of signals that extends from the cell surface to the nuclear transcriptional apparatus is therefore a necessary requirement to put into operation rational therapies that might negate tumor growth (5). This system is governed at the initiation level by mitogens, growth factors, and by negative growth factors, which together determine the expression of effector molecules (which are basically the same in both normal and cancer cells but are expressed differently in measure and time in cancer cells). Exploiting the differential dependence on growth and survival signaling

From: *Apoptosis, Cell Signaling, and Human Diseases: Molecular Mechanisms, Volume 1*
Edited by R. Srivastava © Humana Press Inc., Totowa, NJ

between cancer and normal cells would create therapeutic opportunities to selectively eliminate cancers. Several strategies have been developed based on the identification of molecular lesions that are responsible for deregulated cell replication or molecular lesions that foster cell survival (9–18). Drugs designed to affect identified targets, an approach emphasized within the past decade, are mostly based on inhibitors of the Ras-Raf-MEK-ERK pathway (4,16,17,19–22) and inhibitors of the PI3K/Akt pathway (23–25), but it is now clear that these efforts have not been rewarded by satisfactory clinical success. A main obstacle in many cancers is the multiplicity of genetic lesions that, through different routes, promote replicative growth and protect cancer cells from apoptotic death, thus hampering the effectiveness of drugs directed at single targets. Other likely impediments are the ability of intracellular signaling to integrate and bypass individual blocks, the possible molecular promiscuity of the targeting compound, lack of cell specificity, and the toxicity implicit to the nature of the cell permeable low-molecular-weight pharmacological inhibitors used in these studies. Contrary to this scenario there is suggestion, based both on theoretical discrepancy between incidence of cancer and calculation of mutation probability (26) and on experimental evidence, that in the healthy organism a shift to hyperproliferation can be resolved by the activation of a molecular program which leads to the death of the aberrant cell (*see* Subheading 4.2.). The exploitation of the differential dependence on apoptosis between cells with deregulated growth and normal cells in the healthy organism can only be the prerogative of a naturally occurring molecule whose physiological function is to control cell replication by inducing regulatory changes to which normal and cancer cells respond differently (26). This property, which implies surveillance, suggests that such a molecule could be used within a therapeutic context. We have identified one such molecule in the β -galactoside binding protein (β GBP) (27), which we have cloned and found to be an antiproliferative cytokine (28) which negatively controls the cell cycle in normal cells while selectively activating apoptosis in cancer cells.

2. General Properties of β GBP

Produced by $CD8^+$ and $CD4^+$ activated T-cells (*see* Fig. 1) β GBP is a cytokine (28) with an important role in the silencing phase of T-cell immune response and in the switching off of T-lymphocyte effector functions (29). β GBP is also released by somatic cells where its physiological autocrine function is to negatively control the cell cycle from S phase to G2/M transition (27). β GBP is a 15-kDa monomeric globular molecule whose structural gene (Lgals 1) (30) is located in the sis/PDGFB homology region (mu-chromosome 15 E/Hu-chromosome 22 q12–q13) (31), a syntenic group which undergoes deletions and translocations in a number of human tumors (32–35). Previously classified as a lectin (galectin1), β GBP has no lectin properties. It has only one saccharide-binding site located in the region spanning from residue 64 to residue 76. At concentrations of about $10^{-4}M$ the β GBP molecules can be reversibly held in pairs by hydrophobic interactions becoming transient opportunistic dimers (36), but in its native state β GBP is a monomer which exerts its function as a monomer. Hu-r- β GBP exerts its functions as a negative cell-cycle regulator and as an anticancer agent at concentrations of about $1\text{--}20 \times 10^{-9}M$ (26,27,37–40), several orders of magnitude lower than the transient dimeric form. Hu-r- β GBP binds with high affinity (K_d $1.5 \times 10^{-10}M$) to about 5×10^4 cell surface specific receptors/cell through molecular domains other than those that link saccharide determinants (27). This is demonstrated by sugar competition studies (27,29), by a native form of the molecule where

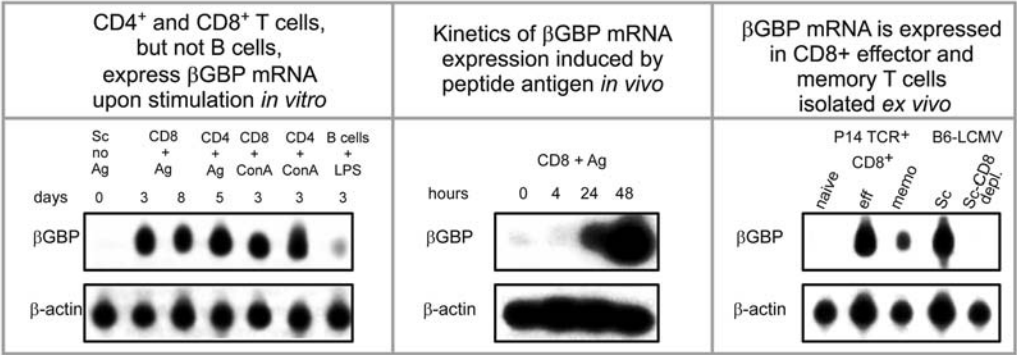


Fig. 1. Northern blot analysis of βGBP mRNA expression. *Left:* CD8⁺ and CD4⁺ T-and B-cells activated in vitro. *Lane 1:* spleen cells from naive C57BL/6 mice; *lanes 2 and 3:* CD8⁺ T-cells from P14 TCR-transgenic mice after peptide stimulation; *lane 4:* CD4⁺ T-cells from 2B4 TCR-transgenic mice 5 d after peptide stimulation; *lanes 5 and 6:* CD8⁺ and CD4⁺ T-cells from C57BL/6 mice 3 d after Con A activation; *lane 7:* B-cells activated with LPS. *Center:* CD8⁺ T-cells after peptide antigen treatment in vivo. P14 TCR-transgenic mice injected with 500 ug LCMV GP 33 peptide ip. *Lane 1:* no peptide; *lanes 2–4:* 4, 24, and 48 h after peptide treatment. *Right:* CD8⁺ T-cells ex vivo from P14 TCR-transgenic mice. *Lane 1:* uninfected; *lane 2:* 8 d after LCMV infection; *lane 3:* 4 wk after LCMV infection; *lane 4:* spleen cells from 8-d LCMV immune C57BL/6 mice; *lane 5:* CD8⁺ T-cell-depleted spleen cells. Reprinted with permission from ref. 28.

the saccharide-binding region is masked by a glycan complex and by the internalization of the saccharide-binding site when βGBP molecules are linked in a tetrameric complex (41). These are all conditions which have no effect on the strictly dose related antiproliferative efficacy of the βGBP molecule which, via receptor interaction, negatively regulates the cell cycle in normal cells and selectively activate apoptosis in cancer cells (26,27,37–40). Though undoubtedly originating within the lectin family, βGBP has evolved to become a cytokine making the term galectin a misnomer.

3. Role in Cell-Cycle Regulation

In normal cells, βGBP operates within the signaling context simplified in Fig. 2 to activate an S-phase checkpoint (see Fig. 3) which imposes a statutory cell-cycle pause before commitment to division. Downregulation of Ras-GTP loading by Hu-r-βGBP and consequent downregulation of the kinase sequence downstream of Ras in primary mouse embryo fibroblasts (MEF) results in reduction of cyclin-A levels, reduction of cyclin A-kinase activity, reduction of E2F function, and S/G2 cell-cycle arrest (42). Negation of the Hu-r-βGBP by a neutralizing monoclonal antibody reverses S-phase arrest and promotes cell-cycle completion (27). The importance of βGBP in the regulation of S/G2 transition is made evident by the fact that neutralization of the endogenous, constitutively released βGBP accelerates transition into G2/M, highlighting the existence of a programmed cell-cycle pause which may permit cells to correct errors which may cause genome instability and growth disorders. In addition to controlling an S-phase checkpoint, βGBP can hold cells in G0 by inhibiting the activation of the Ras-ERK module without affecting tyrosine kinase (TK) receptor phosphorylation. An analysis of the events involved shows that whereas Ras-GTP loading is inhibited in the presence of βGBP, Grb-2 recruitment to the receptor, Grb-2-SOS association and the ability of SOS to activate Ras in vitro are

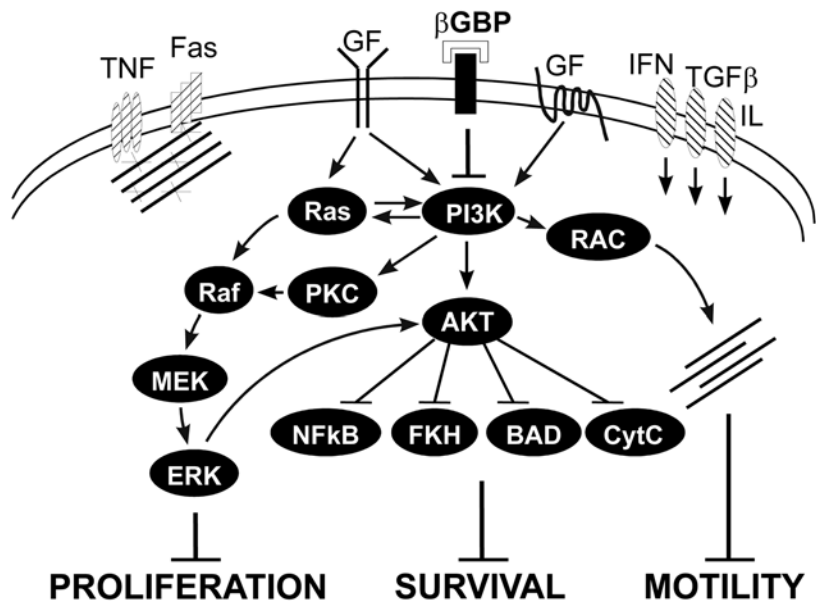


Fig. 2. Schematic model of principal signaling pathways initiating at the cell surface by ligand/receptor interaction and the resulting inhibitory effects of PI3K inhibition by β GBP induced signaling. Abbr: GF, growth factors; IFN, interferon α/β ; TGF- β , transforming growth factor β ; TNF, tumor necrosis factor; IL, interleukins.

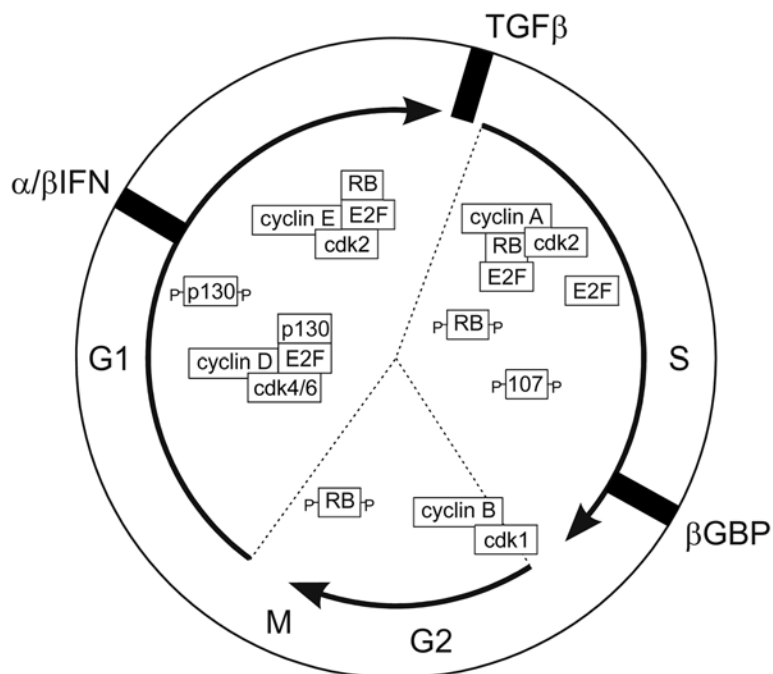


Fig. 3. Cyclin-cdks and E2F-RB/p107 associations in the course of regulatory events which control cell-cycle transitions and location of checkpoints (black bars) activated by IFN α/β (96), TGF- β (97,98), and β GBP (27).

maintained. Also, Ras-GAP activity is not altered. Instead, lack of Ras-GTP loading in the cells treated with Hu-r-βGBP is a consequence of the inactivation of the p110 catalytic unit of phosphoinositide 3-OH kinase (PI3K) (43). These responses make PI3K a prime target of βGBP induced signaling and position PI3K upstream of Ras.

4. Pathways to Apoptosis—Two Molecular Phenotypes

4.1. Molecular Phenotype I

The ability of βGBP to arrest normal cells in S phase, albeit reversibly because normal cells resume growth, extends to a number of tumor cells which, unlike normal cells where cell arrest is a temporary event, undergo programmed cell death. The mechanisms that can activate apoptosis in cancer cells are multifold. Apoptosis can be induced through the modulation of existing apoptosis regulatory proteins and through transcription and translation dependent changes to which normal and cancer cells respond differently (44–50). The apoptotic response induced by βGBP conforms to two main molecular phenotypes. In the case of cancer cells arrested in S phase, ectopic transactivation of the E2F1 transcription factor, when highly expressed, has emerged as one mechanistic aspect (26,51–56). E2F1 activity is required for entry into S phase (57–60); E2F1 downregulation is necessary for S-phase progression and successful cell replication (54,61). Transfection with E2F1 mutants defective in cyclin-A binding leads to S-phase arrest, continuous E2F1 transactivation and apoptosis (53,54). E2F1 can promote apoptosis through p53 dependent and independent pathways (62,63) (see Fig. 4) and can force caspase enzymes to levels that may increase the probability that death inducing signals overcome the ability of inhibitor of apoptosis proteins (IAP) to impede the activation of programmed cell death (64). The importance of E2F1 in the mediation of programmed cell death in cancer was indicated by the finding that loss of E2F1 function by dominant negative mutants in MBL 100 breast cancer cells inhibits apoptosis and induces tumors in severe combined immunodeficiency (SCID) mice (65). By the enforcement of its regulatory functions, βGBP downregulates cyclin A-kinase activity, arrests cells in S phase, shifts E2F1 to a persistent transactivation mode, and activates apoptosis (26,37–39). Cancer cells which respond to βGBP according to this pattern: molecular phenotype I (see Fig. 5) are cells where the ErbB2 oncoprotein receptor is expressed at low levels. They are typified by noninvasive MCF-7 breast cancer cells grown in the absence of estrogens, by p53 defective Ramos lymphoma cells and Ramos cells overexpressing Bcl-2 (26).

4.2. Molecular Phenotype II

Unlike cancer cells which have low ErbB2 levels, in cancer cells where ErbB2 is overexpressed (66–68), apoptosis in response to βGBP does not relate to E2F1 deregulation. A ligandless member of the ErbB/EGF family of TK receptors, ErbB2 strongly enhances Ras regulated Raf-MEK-ERK signaling by being constitutively active, by dimerizing with other ErbB members, by resisting endocytic degradation, and by returning to the cell surface (69–71). ErbB2 downstream effects include cyclin D-kinase activation, activation of DNA synthesis and S-phase progression (72–74). ErbB2 is also a strong activator of class IA phosphoinositide 3-kinase (PI3K) which consists of a 110-kDa catalytic subunit (α,β,δ) and a p85 regulatory/adaptor subunit that plays roles in protein–protein interactions through a series of molecular domains (75). PI3K catalyzes the production of phosphatidylinositol-3,4,5 triphosphate (PIP3) which recruits to the cell membrane Akt/ protein

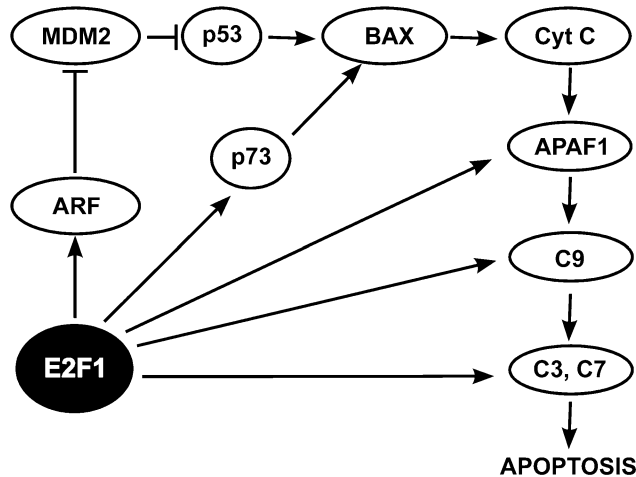


Fig. 4. A composite of pathways reported to be involved in E2F-1 activated apoptosis. Reprinted with permission from ref. 26.

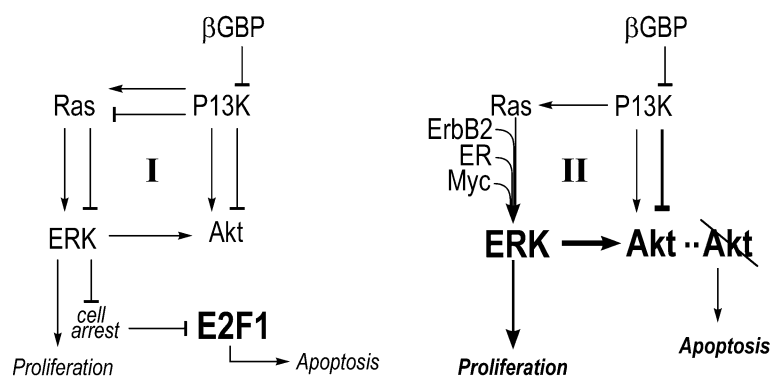


Fig. 5. Models of cancer cells' molecular phenotypes according to their response to treatment with Hu-r-βGBP. Molecular phenotype I is characterized by cancer cells which express high levels of E2F-1 and respond to βGBP by arresting in S phase. In these cells, apoptosis correlates to persistent ectopic E2F-1 transactivation. Molecular phenotype II is characterized by cancer cells where ERK activity is magnified by the converging signaling from multiple sources and PI3K/Akt activity is boosted. PI3K targeting by βGBP and consequent loss of PI3K/Akt signaling deprives the cells of survival capacity.

kinase B (PKB), a key regulator of cell survival (76–78). PI3K also regulates a signaling network that mediates cell growth (79), cell proliferation (80,81), cytoskeletal organization (81,82), cell motility and migration (83–87), all processes of central importance to the evolution of tumorigenesis. Cells that overexpress ErbB2 are characterized by high-ERK levels, fast proliferation rates, and high levels of Akt expression (71,88). In these cells, βGBP is unable to overcome the force of mitogenic signaling but, by targeting PI3K, βGBP abolishes Akt expression and the cancer cells' dependence on survival signaling (42). Loss of Akt is followed after two to three replication cycles by massive cell death. A situation similar to that created by ErbB2 overexpression is also created by estrogen receptor (ER) overexpression, which boosts Ras-ERK signaling (89,90), stimulates Myc activity, induces cyclin D-kinase activity (89,90), stimulates S-phase entry (91,92), and acutely

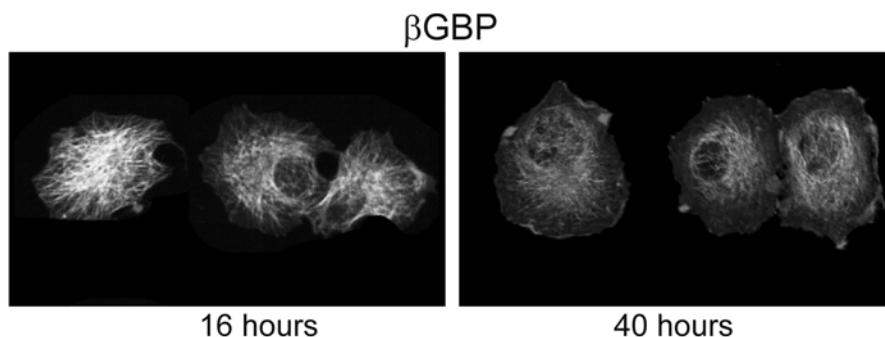


Fig. 6. MCF-7 breast cancer cells treated with β GBP 20 nM undergo cytoskeletal changes characterized by alteration of the tubulin network, loss of tubulin fluorescence, cell spreading, and increased visualization of actin at the edges of the cell membrane. (Photo courtesy of Daniel Zicha, Cancer Research UK.).

increases PI3K/Akt activity (89). Cancer cells which respond to β GBP according to this molecular phenotype II pattern are typified by SKBR3 and BT474 breast cancer cells. Significantly, MCF 10A immortalized mammary ductal cells suffer little damage when exposed to β GBP; however, if their mitogenic input is upregulated by increasing cAMP levels and ERK activity is boosted, their normal-like behavior changes to that of an aggressive phenotype characterized by faster proliferation rate and increased survival properties in terms of Akt phosphorylation. When challenged with Hu-r- β GBP, these cells incur Akt loss and respond with an intensity of apoptotic response similar to that of cells with ErbB2 overexpression. This experimental evidence signifies that where an increase of mitogenic signaling is the prime occurrence among events that lead to oncogenesis, nascent cancer cells could probably be eliminated in the healthy organism by the T-cell produced endogenous β GBP in a surveillance role.

The opening by β GBP of an Akt mediated apoptotic program as a consequence of strong mitogenic signaling whether enforced or endogenous (*see* Fig. 5), does not preclude a prime involvement of PI3K when apoptosis is the result of cell arrest and E2F1 deregulation. Further to the control of cell survival, PI3K plays a critical role in the regulation of S-phase entry via induction of cyclin D expression and activation of cdk 2 and cdk 4 and is necessary and sufficient for E2F activation (93). Inhibition of PI3K results in elevation of p27^{Kip} CDKI levels and cell-cycle arrest (93,94). The inhibition of PI3K by β GBP adds also an extra dimension to the role that β GBP can play in the control of cancer. PI3K is critical to cytoskeleton dynamics and cell motility through the involvement of small GTPases with RAC as a primary regulator (75,81,95). In MCF-7 breast cancer cells PI3K targeting by β GBP leads to loss of tubulin fluorescence, tubulin retraction, cell spreading, and accumulation of actin at the edges of the cell membrane (*see* Fig. 6). Loss of cytoskeletal function and motility is an important aspect in cancer control as it impedes cancer cells from leaving their site and metastasizing. Loss of cytoskeletal function can also cause clustering and activation of cell death receptors to induce apoptosis.

5. Therapeutic Prospects

Hu-r- β GBP has therapeutic efficacy on a wide range of cancer cells of both epithelial and mesenchymal origin while not harming normal cells including the cells of the

Table 1
Effect of Hu-r-βGBP 1–20 nM According to Cell Type
(From Mallucci et al. Biochem Pharmacol 2003;66:1563–1569)

Replication	Apoptosis	
Normal cells	Carcinomas	Lymphomas/leukemias
Luminal breast cells from mamoplasties examined in parallel with BT20 and T47D	Breast BT20, T47D, BT474, SKBR3, MCF-7	ST4, PF382, Jurkat, Raji, JM1, Daudi Ramos p53 [−] Ramos p53 [−] /Bcl-2 ⁺
Expanding T-cells from volunteers examined in parallel with ST4, PF382, and Jurkat	Tamoxifen resistant MCF7/TX9 Multidrug resistant MCF7/D40	U937 Topotecan resistant U937/RERC
Progenitor cells examined in clonogenic assays in parallel with cells from CML patients at diagnosis	Ovarian 2008 Cisplatin resistant 2008 Prostate LNCaP, DU145, PC3 Colon CCL233, SW480, SW620, LoVo, HT29 Colchicine resistant HT29/VMDR Oral KB-3-1 Vinblastine resistant KB-3-1	CML K562, BV173 LAMA-84, KY01 CML from patients at diagnosis

immune system (Table 1). The ability of βGBP to selectively kill cancer including the cells of the immune system cells by opening opportunistic and alternative routes to apoptosis is a unique property not shared by conventional anticancer drugs nor by newly designed pharmacological inhibitors. As an effector molecule which enforces its function within a physiological context, βGBP induces regulatory changes to which normal and cancer cells respond differently because of their differential expression of molecular circuitries whose response to physiological challenges can harm cancer cells. These properties are of major therapeutic significance as they make unusable the acquired means of resisting therapeutic challenges that drug resistant cells have developed (40). Hu-r-βGBP is as therapeutically effective in cancer cells which overexpress P-glycoprotein (MCF-7/D40, KB-V-1, HT-29), in cancer cells with increased DNA repair and metallothionine overexpression (2008/CP), or with altered expression or mutation of topoisomerase I (RERC) or II (MCF-7/D40, RERC), as it is against their parental counterparts (see Fig. 7). There are currently no therapeutic drugs in the clinic that combine

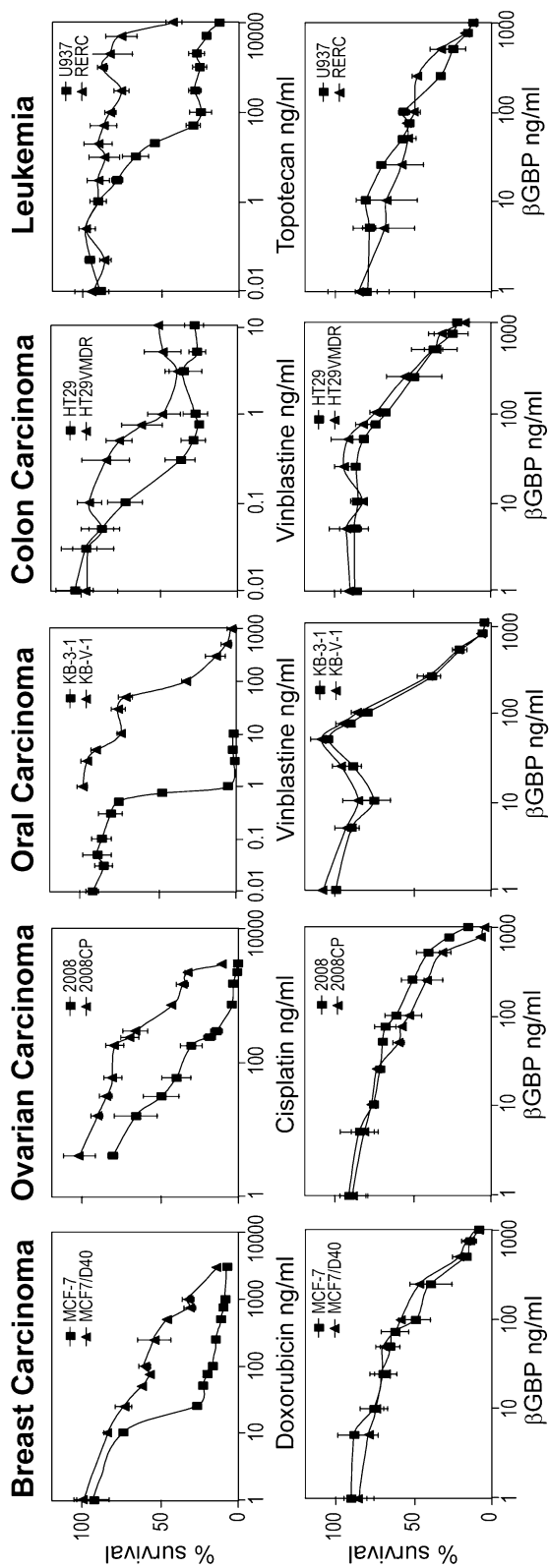


Fig. 7. βGBP bypasses drug resistance. Wild-type cells and their drug-resistant derivatives were treated with either the chemotherapeutic drugs that they were made resistant to or with Hu-r-βGBP and the deoxinucleotidyl transferase dUTP-biotin nick labeling assay performed 72 h post treatment. Reprinted with permission from ref. 40.

the broad spectrum of antitumor activity with the ability to overcome multiple mechanisms of drug resistance as shown by β GBP.

6. Conclusion

β GBP is an immunomolecule and a conceivable new means by which the immune system may control malignancy. The ability of β GBP to perform therapeutically what it would perform naturally within a healthy organism offers a potentially safe and novel therapeutic approach to the control of cancer.

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Control Nodes Linking the Regulatory Networks of the Cell Cycle and Apoptosis

Baltazar D. Aguda, Wee Kheng Yio, and Felicia Ng

Summary

Depending on the nature of extracellular stimuli and the ensuing intracellular signal transduction pathways, certain transcription factors are activated and subsequently determine the extent of expression of genes involved in cell proliferation, survival, and death. These factors are referred to as transcriptional control nodes because they permit the coordination of cell-cycle progression and the apoptosis program. This coordination is made possible by the existence of feedback loops in the regulatory networks of the entire system. A review of these networks for the following transcription factors is provided in this chapter: E2F, Myc, p53, and NF- κ B.

Key Words: E2F; Myc; p53; NF- κ B; cell cycle; apoptosis; regulatory networks; control nodes; modules.

1. Introduction

It is not surprising that the molecular regulatory networks of the cell-division cycle and apoptosis are tightly intertwined ([1](#)) because when the so-called cell cycle engine “overheats” in certain cells in an organism, a mechanism has to exist to get rid of these cells in order to steer a normal course of development or maintenance of the organism. Thus, it is quite reasonable to expect that at least one pathway leading to the initiation of the cell-suicide program is linked to at least one pathway leading to the initiation of the cell cycle. Without such link the coordination between the cell cycle and apoptosis machineries cannot be controlled. In this chapter, it is postulated that the key links are provided by certain transcription factors (TFs) controlling the expression of genes involved in the cell cycle and apoptosis. Activation of these TFs is initiated by extracellular signals transduced by intracellular pathways that directly cause the translocation of the TFs to the nucleus, binding to gene promoters and initiation of transcription. These transcriptional nodes are referred to in this chapter as “control nodes” because they permit the possibility of coordinating the cell cycle and apoptosis. The discussion is organized according to the general classification of signaling pathways and control nodes shown in [Fig. 1](#) ([2](#)). The pathways in this figure should be interpreted in a very general way; for example, type I signaling pathways activate type I control nodes which potentiate pathways promoting the cell cycle and apoptosis (“potentiate” here means that the pathway does not necessarily trigger activation). Note that the signaling pathways shown in [Fig. 1](#) are only those that activate the control nodes but, in general, the discussion will also include activating and inhibiting signaling pathways and their interactions.

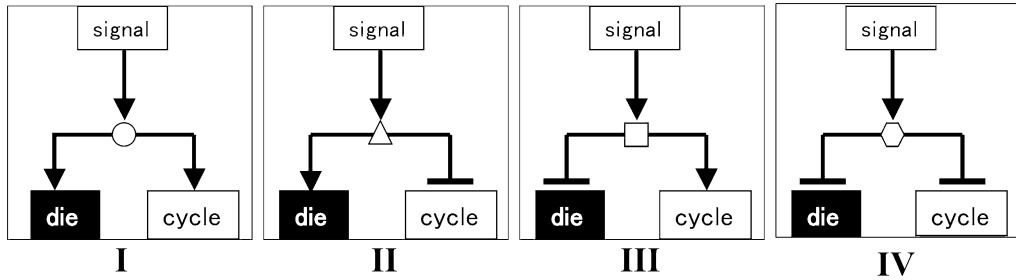


Fig. 1. Transcriptional nodes (symbolized by a circle, a triangle, a square, and a hexagon) are classified according to how they affect the cell cycle and apoptosis. Arrows represent “induction” whereas hammerheads represent “inhibition.” Associated intracellular signalling pathways that activate these nodes are shown.

Furthermore, an important observation is that no case as shown in Fig. 1 would give rise to a mechanism in which the status of the cell-cycle module influences the status of the apoptosis module. Indeed, one can see that there should be at least a pathway from the cell-cycle module which feeds back to the transcriptional node or to any upstream signaling pathway that regulates the TF node activity; this feedback loop would generate the situation in which the TF control node activity is a function of the cell-cycle module status (i.e., the activity of some cell-cycle factor) and therefore the status of the apoptosis module would be an indirect function of the cell-cycle module status. Of course, the cell-cycle module can directly communicate to the apoptosis module by either regulating the pathways from the TF node to apoptosis and/or the apoptosis module itself.

The known molecular regulatory networks of the cell cycle and apoptosis are quite complex and can overlap. However, as suggested in Fig. 1, it will be assumed that these networks are separable and can be modularized by focusing on the primary enzymes that trigger these cellular processes, namely, the cyclin-dependent kinases (CDKs) for the cell cycle and the caspases for apoptosis. A discussion of the basic components and interactions in these modules will be provided in the next section. As will be shown, these modules represent tightly regulated interactions that communicate with the transcriptional nodes in both directions.

The control nodes discussed in depth in this chapter include the E2F, Myc, p53, and NF- κ B transcription factors. E2F and Myc are combined into one node which will be referred to as the E2F-Myc node; E2F here specifically represents E2F-1 (a member of the E2F family) and Myc refers to c-Myc. These TFs are grouped together because of their synergistic interactions and the fact that they possess many common transcriptional targets. The E2F-Myc node represents case I in Fig. 1. The tumor suppressor function of the protein p53 is mainly attributed to the proapoptotic gene targets; in addition, p53 induces the expression of several genes whose protein products inhibit cell-cycle progression. Thus, p53 is an example of case II in Fig. 1. Case III is exemplified by the NF- κ B family of dimeric transcription factors which is primarily known for its role in the immune response, but is now increasingly linked to carcinogenesis through pathways that connect to the cell cycle and apoptosis (case IV in Fig. 1 is not of interest in this chapter). Although there are more transcriptional nodes that can control the

cell cycle and apoptosis programs, the above examples are deemed sufficient to illustrate some of the principles needed to understand the operation of transcriptional control nodes as they affect the cell cycle and apoptosis modules.

A detailed analysis of the E2F-Myc control node and its interactions with the signaling, cell cycle, and apoptosis modules illustrate some important concepts governing the operation of these networks. There are several coupled positive and negative feedback loops present in the regulatory networks, and these loops operate both at the level of a single module and at the level of module–module interactions. Positive feedback loops could lead to switching and bistable behavior whereas negative feedback loops can generate sustained oscillations. The structures of the regulatory networks of the other transcriptional nodes in terms of feedback loops are therefore discussed to demonstrate how sources of switching and other unstable behaviors are predicted. The intrinsic nonlinearities of these control networks make them very difficult to understand intuitively. Indeed, mathematical modeling and computer simulations are tools that would greatly aid in understanding these complex networks.

2. Cell Cycle and Apoptosis Modules

2.1. G1/S Module

Based on a kinetic analysis of the then prevailing consensus G1-S regulatory network of the mammalian cell cycle, Aguda and Tang (3) proposed that the G1 checkpoint called the “restriction point” is governed by the activation of cyclin E/CDK2 whose critical regulators include the phosphatase Cdc25A, and a CDK inhibitor (p27Kip1). This picture has not changed much as can be seen in a recent review of the field (for an example, *see ref. 4*). However, recent experiments with mice knocked out of G1 cyclins and G1 CDKs suggest that the various mammalian cyclins and CDKs may take each others roles if necessary (5,6).

According to the Aguda-Tang kinetic analysis, sharp switching behavior in CDK2 activity is predicted from the mutual-activation (between CDK2 and Cdc25A) and mutual-inhibition (between CDK2 and p27Kip1) as shown in Fig. 2. Cdc25A removes an inhibitory phosphate from CDK2 thereby promoting the kinase activity of the latter; in return, the phosphatase activity of Cdc25A is upregulated by its phosphorylation carried out by CDK2. Such positively coupled cycles of phosphorylation and dephosphorylation possess an intrinsic instability that generates switching behavior (7); this switch is further sharpened when coupled to the mutual antagonistic interactions between p27Kip1 and CDK2. Several experimental observations are consistent with the Aguda-Tang model (8–10).

In quiescent cells, members of the retinoblastoma protein (pRB) family bind and inhibit proliferative transcription factors such as the E2Fs which are essential for the expression of several S-phase genes. Growth factors stimulate the synthesis of the D-type cyclins which bind and activate CDK4 and CDK6; these kinases then phosphorylate and inactivate pRB subsequently freeing E2F to induce the expression of cyclin E, cyclin A, Cdc25A, several members of the origin recognition complex (ORC). Cyclin E/CDK2 and cyclin A/CDK2 further contribute to the hyperphosphorylation of pRB (for review, *see ref. 11*). The time lag in the activation of cyclin E/CDK2 after crossing the restriction point (3,8) could be explained by an induction time associated with the build up of the positive feedback loop involving pRB, E2F, and cyclin E/CDK2 (*see Fig. 2*).

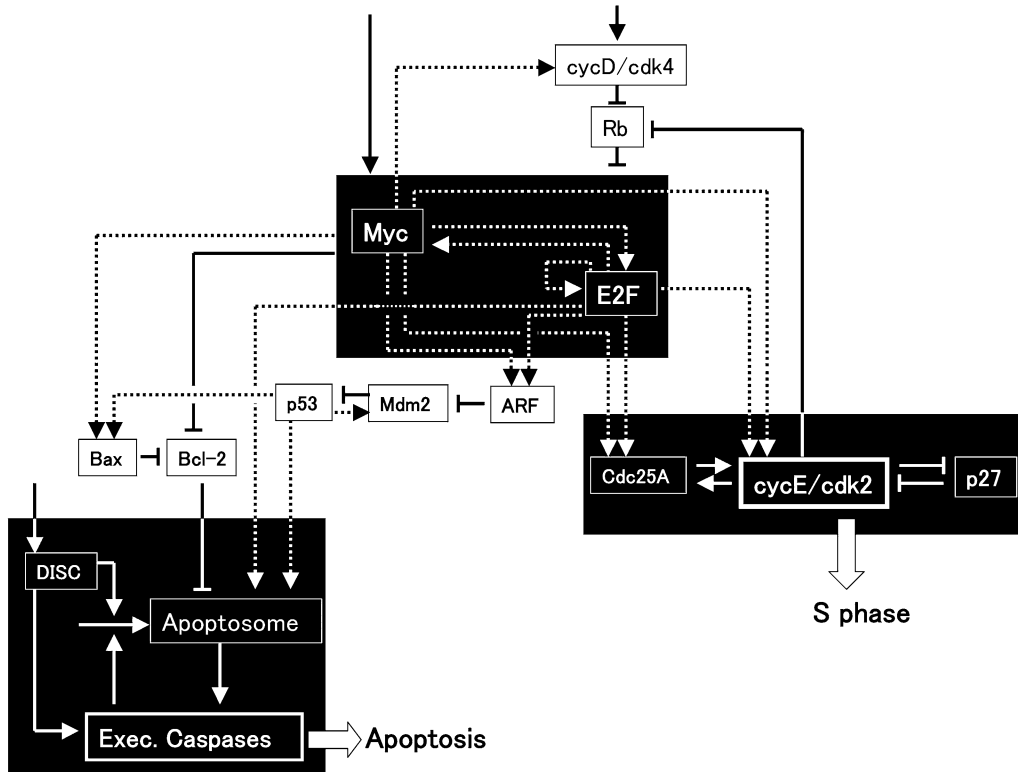


Fig. 2. The E2F-Myc node is the top-most black box which encapsulates the synergy between the E2F and Myc transcription factors. Dotted lines indicate transcriptional activation (with arrow heads). Regulation of the E2F-Myc node activity includes both Rb-dependent and Rb-independent pathways as represented by the two signaling routes shown. The right-most black box represents a minimal module for the regulatory network involved in CDK2 activation in S-phase entry. Solid lines mean posttranscriptional interactions with an arrow indicating “activation” and a hammerhead denoting “inhibition.” The left-most black box represents the apoptosis module involving cascades of activation of the caspases.

2.2. Apoptosis Module

The apoptosis module involves cascades of activation of proteases of the caspase family (for a recent review, *see* ref. 12). Members of this family include the initiator caspases (caspase-8, -9, -2, -10) and executioner caspases (caspase-3, -6, -7). The two major pathways that converge at active executioner caspases are summarized in Fig. 2 and in ref. 2. The extrinsic or membrane receptor-mediated pathway involves a complex called death-inducing signaling complex (DISC) which forms upon ligand-receptor binding (e.g., tumor necrosis factor [TNF]- α and FasL ligands binding to corresponding receptors). For example, the accumulation of procaspase-8 in the DISC generates active caspase-8 molecules which are released from the complex to activate the executioner caspases. The intrinsic or mitochondrial pathway involves the formation of the “apoptosome,” a complex composed of cytochrome *c*, Apaf-1, and procaspase-9. As a result of internal cellular stresses (e.g., DNA damage, reactive oxygen species [ROS], growth factor withdrawal) proapoptotic proteins (e.g., Bax, Bad) induce permeabilization of the mitochondria and subsequent release of

cytochrome *c*. Formation of the apoptosome is followed by the activation of caspase-9 which promotes the activation of the executioner caspases. As shown in the apoptosis module in Fig. 2, the extrinsic pathway feeds into the intrinsic pathway (e.g., via activation of Bid by caspase-8; Bid induces release of cytochrome *c* from mitochondria); moreover, a positive feedback loop is shown from the executioner caspases to the step that forms the apoptosome (representing the observation that executioner caspases induce an increased rate of cytochrome *c* release from mitochondria (13,14). This positive feedback loop is thought to be an essential mechanism for the irrevocable switch into apoptosis (2).

3. The E2F-Myc Node

3.1. Regulation and Targets of the E2F-Myc Node

The E2F-Myc transcriptional node that induces expression of several genes involved in S-phase entry as well as genes involved in apoptosis is shown in Fig. 2. E2F dimerizes with DP proteins whereas Myc dimerizes with Max in order to carry out their respective transcriptional activities. E2F-1 has been shown to upregulate c-Myc expression (15–17) and c-Myc has been shown to induce transcription of E2F-1, E2F-2, and E2F-3 (18,19). Thus, E2F-1 and c-Myc synergize to induce S-phase entry and apoptosis (2,20).

A schematic representation of signal transduction pathways impinging on the E2F-Myc node is shown in Fig. 2. One involves a pathway from cyclin D/CDK to the retinoblastoma protein (pRB). Several studies have shown that the Ras family of small GTPases are involved in the upregulation of synthesis or stabilization of some D-type cyclins (11). These cyclins bind and activate CDK4 or CDK6 which ultimately results to increased E2F transcriptional activity (because these CDKs phosphorylate pRB which then releases E2F). The significance of studying the pRB pathway is underlined by the fact that it is targeted for inactivation in at least 80% of sporadic human cancers (21). Chau and Wang (22) recently reviewed evidence that pRB could be a key protein in the regulation of a cell's life and death decisions. This role of pRB is directly associated with its inhibition of the E2Fs. The interplay between mitogenic signals and death signals (e.g., TNF signaling) uses pRB as a key player; mitogenic signals inactivate pRB by phosphorylation via CDK activation, whereas death signals inactivate pRB by caspase-dependent degradation (for a review, see ref. 22).

A few of the major transcriptional targets of E2F-1 are shown in Fig. 2. These include S-phase genes such as cyclin E, cyclin A, Cdc25A, and several members of the prereplication protein complex formed prior to DNA replication (reviewed in ref. 11). It is also interesting to note that there is a positive feedback loop involving the E2F-1 protein as a positive transcription factor for its corresponding gene (23). Among the proapoptotic genes induced by E2F-1 are Apaf-1 (a member of the apoptosome complex) and various caspases (24–26). Another E2F-1 target is ARF which indirectly stabilizes p53. E2F-1 can also promote apoptosis, independent of its transcriptional activity, by binding with the p65 subunit of NF- κ B and inhibiting the latter's antiapoptotic functions (27). In the presence of DNA damage, ATM, ATR, and Chk2 kinases have been shown to phosphorylate E2F-1 resulting in the stabilization of this substrate (28).

Note that activation of cyclin D/CDK4/6 is not absolutely required for the activation of cyclin E/CDK2; this is because signaling pathways mediated by c-Myc can upregulate expression of proteins involved in CDK2 activation as well as in the downregulation of the CDK inhibitor p27Kip1 (29). Santoni-Rugiu et al. (29) have demonstrated

that Myc is involved in a G1/S-promoting mechanism that is parallel to the pRB/E2F pathway. Myc is an immediate-early gene that is expressed soon after a cell's exposure to growth factors leading to the activation of tyrosine kinases such as Src and JAK that upregulate Myc (reviewed in ref. [11](#)). Myc's transcriptional targets which are involved in S-phase entry include cyclin D2 ([30,31](#)), CDK4 ([32,30](#)), cdc25A ([33](#)), E2F-2 ([18](#)), and cyclin E ([34](#)). Proapoptotic target genes include ARF and Bax as shown in [Fig. 2](#). The pathway leading to apoptosis is also favored by the suppression of the protein and RNA levels of Bcl-2 by c-Myc as well as by E2F-1 ([35](#)). Furthermore, it has been suggested ([36](#)) that c-Myc promotes apoptosis by repressing the expression of p21Cip1 which indirectly inhibits apoptosis via the mitochondrial pathway ([37](#)).

3.2. Coordinating S Phase and Apoptosis

A major question of interest is how the E2F-Myc node coordinates the initiation of S phase and apoptosis; as an example of this coordination, E2F-1 first stimulates S-phase entry when its transcriptional activity is within some normal range but drives the apoptosis program when overexpressed. Looking at case I in [Fig. 1](#), it is important to remember that the arrow from the E2F-Myc transcriptional node to apoptosis indicates that apoptosis is potentiated and not necessarily triggered; could it be that a trigger for apoptosis is generated by an over-stimulated cell cycle? Is it also possible that the cell-cycle mechanism itself generates a signal that somehow inhibits apoptosis so that the cell is not in danger of accidentally killing itself when everything else is normal? And how do the different cases in [Fig. 1](#) interact to determine cell fate? These questions were addressed in a recent paper by Aguda and Algar ([2](#)). It is proposed in this paper that the complexity of the network of molecular interactions can be reduced by subdividing the network into functional modules and analyzing module-module interactions. These modules and their interactions are shown in [Fig. 3](#). The module-module interactions are labeled **a**, **b**, **b'**, and **c**. Interaction **a** includes positive feedback loops involving the transcriptional node and the S-phase module; examples would be the loop E2F-CyclinE/CDK2-RB-E2F and the autocatalytic loop involving E2F-1. Aguda and Algar ([2](#)) noted the importance of the negative feedback **b** from the cell-cycle module to the signaling pathway upstream of the transcriptional node; as the computer simulations in their paper demonstrate, this negative feedback permits a window or a range of transcriptional activities for normal cell cycles to exist by inhibiting apoptosis. The existence of a negative feedback loop involving the Ras/Raf/MEK/ERK pathway can be supported by experimental evidence ([38–40](#)) of multiple peaks of activities of Ras and/or MEK/ERK; these peaks look like damped oscillations which are indicative of negative feedback in a dynamical system. Another negative feedback is the interaction **b'** between the cell-cycle module and the transcriptional node; the phosphorylation of DP-1 (a partner of E2Fs) by CDK2 can turn off E2F-1 transcriptional activity ([41,42](#)). Lastly, the feedforward type of interaction **c** in [Fig. 3](#) is a direct way of triggering apoptosis when the cell cycle “overheats;” experimental evidence for this interaction has been reported ([43–45](#)).

4. The p53 Node

The p53 protein is a DNA sequence-specific transcription factor that regulates the expression of certain genes associated with the induction of apoptosis, cell-cycle arrest, prevention of new blood vessel formation, and accelerated DNA repair. The induction

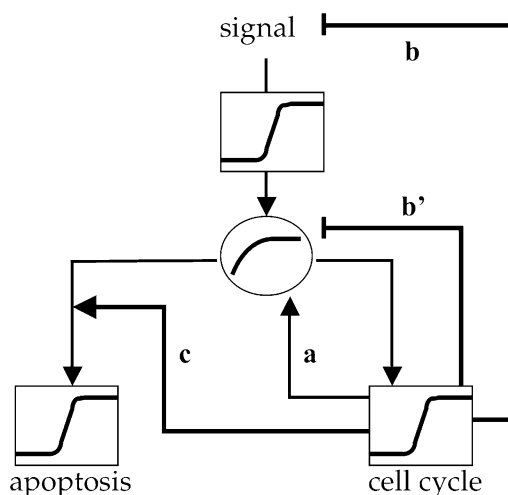


Fig. 3. Case I of Fig. 1 including module–module interactions. *Arrows* indicate activation whereas *hammerheads* indicate inhibition. This is the modular model analyzed by Aguda and Algar (2). The signaling, apoptosis, and cell-cycle modules were assumed to possess ultrasensitive kinetics (as shown by the curves inside the rectangular boxes). The transcriptional node (circle) was not assumed to have that kinetics.

of apoptosis is widely accepted as the primary role of p53 in tumor suppression. The levels of p53 in normal cells are low but rapidly increases upon exposure to DNA-damaging stresses including ultraviolet light and intense oncogenic signaling. The p53 gene is mutated in about half of known human cancers whereas a majority of the remaining cases are caused by dysfunctional regulation of an otherwise normal p53 protein (20,46–48).

The p53 control node shown in Fig. 4 symbolizes a set of active transcription factor complexes involving p53 that directly affect the expression of target genes, some of which are also shown in the figure. Signaling to the p53 node includes all upstream conditions and processes (e.g., exposure to ultraviolet light and presence of DNA damage) that cause elevated rates of p53 synthesis, covalent modifications of the protein, DNA binding and activation of p53-mediated transcription. Post-translational modifications (i.e., phosphorylation, acetylation, sumoylation) that regulate p53 activity are also considered processes signaling to the node. Many details of these regulatory pathways are already known, and comprehensive reviews of the current state of knowledge in the field are available (46,49,50) and will not be duplicated here. The primary aim here is to interpret the significance of these pathways in relation to their role in deciding whether the cell dies or survives. The interpretation is based on significance of the cycles present in the regulatory network. Figure 5 summarizes some of the positive and negative feedback loops in the p53 regulatory network. Harris and Levine (51) recently reviewed the various positive and negative feedback loops in the p53 regulatory network.

4.1. The p53-Mdm2-ARF Network and Negative Feedback Loops

As shown in Figs. 4 and 5, the regulation of p53 activity involves several coupled negative feedback loops; one of these is a two-cycle involving Mdm2 and p53, and another is a three-cycle involving p53, ARF, and Mdm2. In the 2-cycle, Mdm2 binds

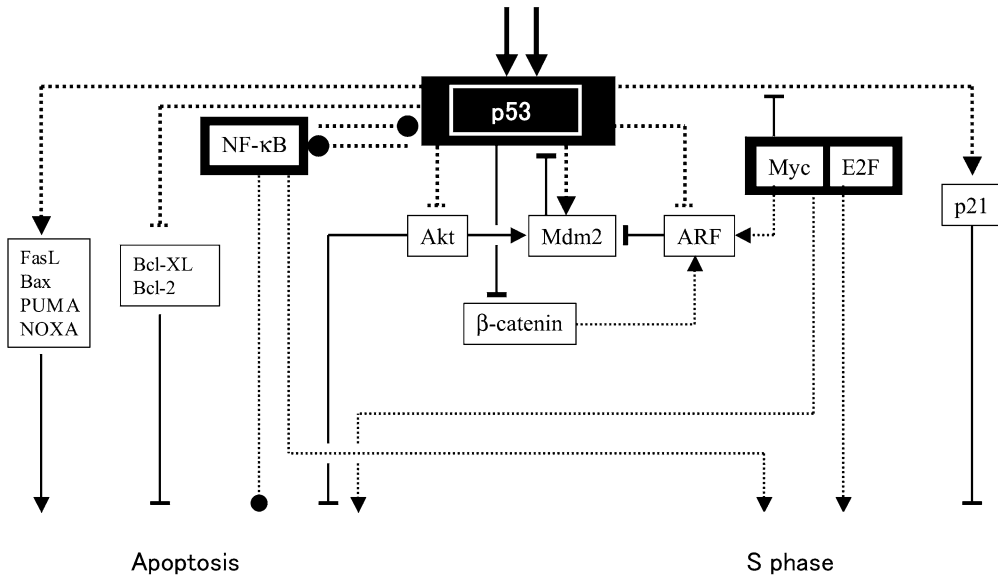


Fig. 4. The p53 transcriptional factor promotes expression of certain proapoptotic genes (e.g., FasL, Bax), of the CDK inhibitor p21, and represses transcription of certain antiapoptotic genes (e.g., Bcl-XL, Bcl-2). The core regulatory network involving Mdm2 and ARF is shown, as well as the links of this network to Akt and β -catenin. Interactions among the NF- κ B and E2F-Myc nodes are discussed in the text. Dotted lines represent transcriptional processes while solid lines are post-translational interactions. Arrows mean “activation” and hammerheads mean “inhibition.”

and inhibits p53 by blocking a transactivation domain of p53 and by promoting the ubiquitination of p53 leading to proteosomal degradation; on the other hand, p53 induces the expression of the *mdm2* gene by binding to this genes promoter region. This negative feedback loop between p53 and Mdm2 has been offered as the origin for observed oscillations in p53 activity (52,53).

The transcription of the p14/19/ARF gene is negatively regulated by p53 (51). In turn, ARF binds to and represses the ubiquitin ligase activity of Mdm2 (54). ARF is not expressed in normal proliferating cells but is rapidly expressed in response to many oncogenes such as c-Myc, E2F-1, Ras, and β -catenin; this is why ARF is considered as one of the most important “sensors for aberrant proliferative signaling” (20).

As shown in Fig. 5, p53 induces transcription of SIAH-1, a ubiquitin ligase that targets β -catenin for degradation (51). A negative feedback loop is thus formed by the pathway represented by the sequence p53-(SIAH-1)-(β -catenin)-ARF-Mdm2-p53. This links the β -catenin signaling pathway to the p53 network. Other negative feedback loops shown in Fig. 5 are p53-p21-CDK2-Mdm2-p53 and p53-(WIP-1)-p38MAPK. The gene p21Cip1, which produces a cyclin-dependent kinase (CDK) inhibitor, is one of the first identified transcriptional targets of p53. As shown in Fig. 5, CDK2 inhibits Mdm2 by phosphorylation; interestingly, this inhibitory phosphorylation is counteracted by the phosphatase PP2A in complex with cyclin G, which is rapidly transcribed after p53 activation in many cell types (reviewed in ref. 51). This gives a picture of Mdm2 regulation by phosphorylation-dephosphorylation steps which are influenced by p53 via p21 (resulting to inhibition of the phosphorylation step) and via cyclin G (which enhances the dephosphorylation step).

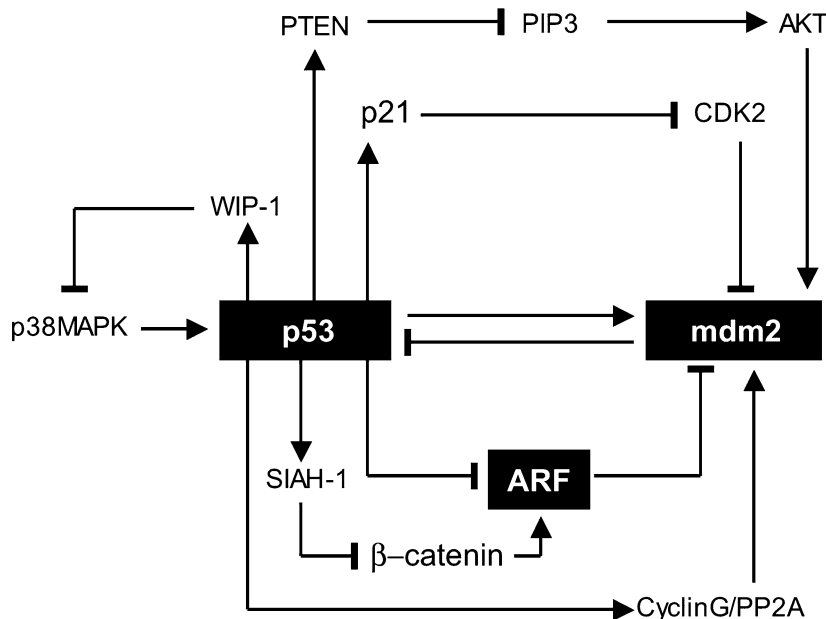


Fig. 5. Some of the feedback loops coupled with the p53-Mdm2-ARF core regulatory network as reviewed by Harris and Levine (51). See text for details.

The Ras/MAPK signaling pathways are linked to the p53 regulatory network as shown in Fig. 5. Activation of p53 partly involves its phosphorylation by p38MAPK on certain serine residues (reviewed in ref. 51). The protein p38MAPK itself is activated by phosphorylation which is reversed by the Wip-1 phosphatase.

4.2. Positive Feedback Loops in p53 Regulation

Shown in Fig. 5 is a positive feedback loop represented by the sequence p53-PTEN-PIP3-Akt-Mdm2-p53. PTEN is considered a tumor suppressor whose transcription is induced by p53 in some cell types (reviewed in ref. 51). PTEN is a lipid phosphatase that dephosphorylates PIP3 (phosphatidyl inositol-3,4,5-triphosphate). PIP3 is required for the recruitment of Akt to the plasma membrane where it is phosphorylated and activated. In addition, p53 represses the catalytic subunit of PI3K (phosphatidylinositol 3-kinase) which catalyzes the formation of PIP3 (55). Akt is a survival factor because it phosphorylates and subsequently inhibits proapoptotic proteins such as Bad and caspase-9. Akt also phosphorylates Mdm2 which subsequently translocates to the nucleus where it inhibits p53. The positive feedback loop between p53 and Akt is of the mutual antagonism kind: high p53 activity (apoptosis) means low Akt activity (low survival), and vice versa. Indeed, such mutual antagonism could provide a mechanism for switching between the two cell fates of survival and death.

The picture becomes more complicated when the participation of another important kinase, glycogen synthase kinase-3 β (GSK-3 β) is considered. The substrates of this kinase regulate cell proliferation and metabolism (56,57). Shown in Fig. 6 are the interlocking pathways of p53, Akt, GSK-3 β , and β -catenin. This figure shows various positive feedback loops in addition to that between Akt and p53 discussed in the preceding

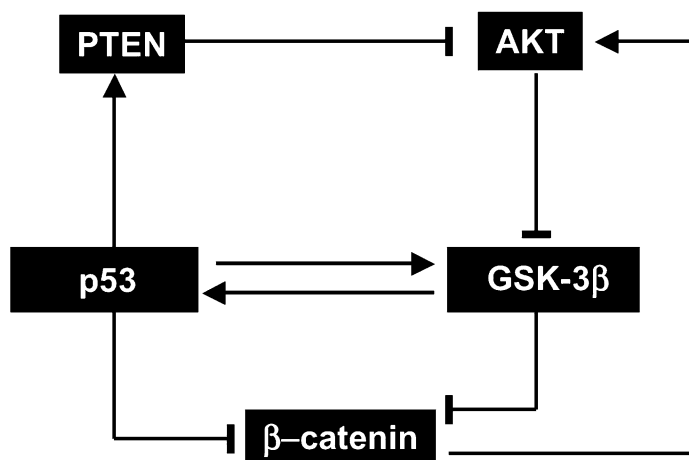


Fig. 6. Some of the positive feedback loops involving p53. Mutual antagonism between p53 (proapoptotic) and Akt (prosurvival), involving PTEN and GSK-3 β , could provide a switching mechanism between cell survival and apoptosis. On the other hand, repression of β -catenin by p53 and GSK-3 β could reduce β -catenin-Akt mediated tumorigenesis. *See text for more details. Modified from ref. 48.*

paragraph. GSK-3 β and p53 have been shown to interact directly (58) and that this interaction augments the activities of both proteins (48) (Fig. 6, double arrows). GSK-3 β phosphorylates and subsequently inactivates proliferative factors such as cyclin D, c-Myc, and β -catenin, among others. True to its role as a survival factor, Akt can phosphorylate and inactivate GSK-3 β as this kinase has been shown to trigger apoptosis when in excess (59). The pathway of activation of Akt from β -catenin could involve increased expression of Wnt-induced secreted protein (WISP)-1 (reviewed in refs. 4,60).

The question remains regarding the role of the positive feedback loops involving p53. It was stated above that the positive feedback between Akt and p53 is a good candidate mechanism for a switch between survival and apoptosis. The positive feedback loop between GSK-3 β and p53 could be a mechanism for favoring the apoptosis pathway. The positive loop (*see* Fig. 6) is another candidate for a switching mechanism between survival and death because it could be interpreted as a toggle switch between Akt (survival) and GSK-3 β (apoptosis). The interactions among these positive loops (as well as between negative- and positive-feedback loops) are difficult to understand using mere intuitive reasoning and it would be interesting to see what computational modeling can contribute to future understanding of this complex network.

4.3. Are There Critical Determinants of p53-Mediated Decision on Life and Death?

If there is a critical determinant of a p53-mediated decision on life or death of a cell, it will surely depend on the cellular context such as the presence of survival signals and cell genotype (48). For example, Myc has been suggested as a critical determinant between p53-mediated cell-cycle arrest and apoptosis (47). Without Myc, cells exposed to ultraviolet light undergo growth arrest primarily as a result of the activation of the CDK inhibitor p21 (61,62). Myc (via recruitment by Miz-1) effectively blocks p21

induction by p53 and tilts the decision towards apoptosis instead of growth arrest (63). In human colorectal cancers, the balance between p21 and PUMA appears to be critical in deciding to arrest or die in response to exogenous p53 (reviewed in ref. 47). Another possible critical determinant is E2F-1 which cooperates with p53 in various ways (e.g., by direct E2F1-p53 association), and by binding to adjacent promoter sites of proapoptotic genes as best illustrated by the case of Apaf-1 (24). The authors of this chapter think that identification of critical determinants of p53-mediated life or death decisions will ultimately depend on understanding the feedback loops between p53-induced apoptotic pathways and survival pathways (e.g., the Akt pathway).

5. The NF- κ B Node

5.1. Regulation and Targets of the NF- κ B Node

NF- κ B is a family of inducible transcription factors that are key responders to changes in cellular environment (64). They play their most important role in the immune system by producing chemokines that trigger innate immune responses. The family consists of p50 (NF- κ B1), p52 (NF- κ B2), p65 (RelA), RelB, and c-Rel. These members form dimers in various combinations in order to carry out their transcriptional activities. The prototypical NF- κ B complex is the heterodimer p50/p65. Each member protein contains a Rel-homology domain that is involved in binding to a DNA sequence motif called the κ B site, in dimerization with other family members, and in binding to I κ B which inhibits nuclear translocation of NF- κ B. Some of the transcriptional targets of NF- κ B are involved in cell proliferation (e.g., cyclin D1 and Myc), cell survival (e.g., Bcl-X_L, IAPs), angiogenesis (e.g., vascular endothelial growth factor [VEGF], IL-8), and the immune response (e.g., major histocompatibility complex [MHC]-I, MHC-II). Malfunction of NF- κ B has been implicated in various hematological disorders, breast cancer, and many other cancers and human diseases (65,66). Connections between inflammation and cancer have also been reviewed recently (67,68).

The canonical pathway of NF- κ B activation is initiated by upstream signals that activate a macromolecular complex called the “signalsome” (69). The upstream signals include cytokines (e.g., TNF α , LPS, CD40L), reactive oxygen species, viral and bacterial products (70). The “signalsome” is an IKK complex composed of IKK α , IKK β , and IKK γ (NEMO). IKK β is the principal kinase in the signalsome that phosphorylates I κ B leading to the latter’s degradation; free NF- κ B then rapidly translocates to the nucleus.

Noncanonical pathways are initiated by agonists such as CD40L, BAFF, and RANKL that induce the activation of a protein kinase called NIK (NF- κ B inducing kinase) (64,70). NIK phosphorylates a dimer of IKK α which initiates proteolytic processing of p100 to p52. Subsequently, p52 along with its dimer partner (principally RelB) translocates to the nucleus.

Signaling to the NF- κ B node involves both positive- and negative-feedback loops as shown in Fig. 7. The negative feedback is results from NF- κ B inducing transcription of I κ B which, in turn, inhibits NF- κ B (71–73); more specifically, inside the nucleus, the newly synthesized I κ B α protein binds NF- κ B resulting to the export of the complex out of the nucleus (74,75). Interestingly, NF- κ B is also known to upregulate the synthesis of proteins that activate NF- κ B, thus forming a positive feedback loop (see Fig. 7); examples are the proteins activating upstream signals of the canonical pathway such as TRAF1/2 (76), CD40 (77), and CD40L (78).

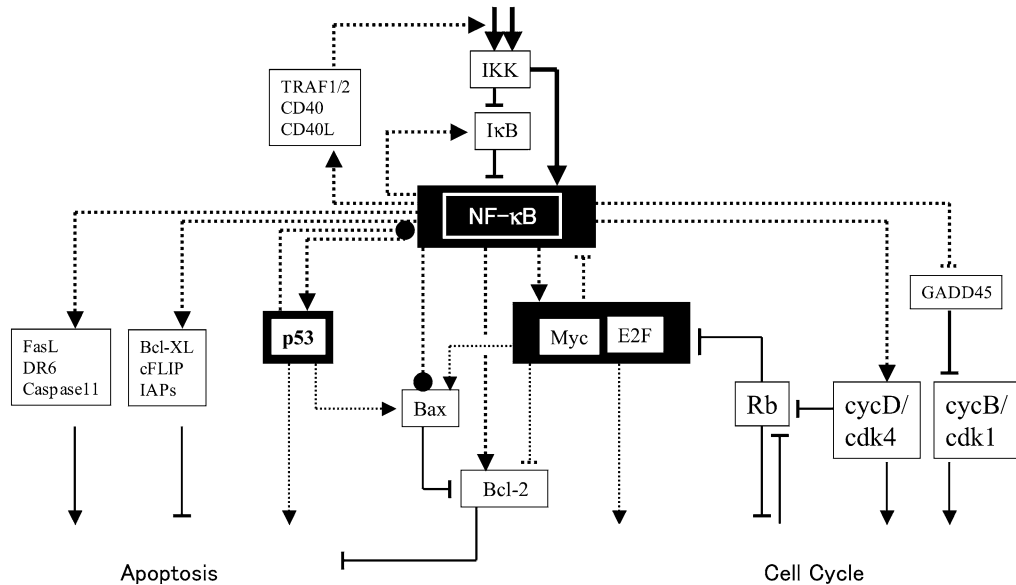


Fig. 7. Overview of the NF- κ B control node. Upstream stimuli activate NF- κ B via two groups of pathways. In the canonical pathway, activated IKK complex triggers degradation of I κ B leading to activation of NF- κ B; noncanonical pathways involve NIK activation of IKK α dimer to produce NF- κ B heterodimers. NF- κ B-induced transcription of I κ B generates a negative feedback whereas NF- κ B-induced transcription of TRAF1/2, CD40, and CD40L results in positive-feedback loops. As shown on the left-hand side of the diagram, NF- κ B may induce expression of both antiapoptotic and apoptotic genes. The role of NF- κ B in the cell cycle is exemplified by promoting the expression of genes such as cyclin D and gadd45 as shown on the right-hand side of the figure. See text for more details.

5.2. Antiapoptotic, Proapoptotic, and Proliferative Pathways From NF- κ B

NF- κ B protects the cell from apoptosis through induction of antiapoptotic genes such as Bcl-X_L (79), Bfl-1/A1 (80), cFLIP (81), and IAPs (76,82). These genes inhibit apoptosis in various ways. Examples include inhibitors of apoptosis (IAPs) inhibiting caspase-3, -6, -7 and -9; cFLIP inhibiting the apoptosome; and Bcl-2 inhibiting the release of cytochrome *c* from mitochondria.

On the other hand, NF- κ B has been shown to target proapoptotic genes such as FasL (83), DR6 (84), and caspase-11 (85). Characteristics of the activating stimulus of NF- κ B may determine whether antiapoptotic or proapoptotic genes would be induced (86). Interestingly, both the pro- and antiapoptotic functions of NF- κ B could occur in the same cell (87), and it may well be that the role of NF- κ B in a cell's decision to live or die is the net result of these opposite apoptotic functions. What then determines the net effect of NF- κ B on apoptosis? Differential regulation of apoptosis between different member proteins of the NF- κ B family is possible (e.g., p65/p50 inhibits transcription of the Bax gene while p50/p50 upregulates it [88]). In addition, relationships between NF- κ B family members and apoptotic p53 may play some role. There is evidence showing that NF- κ B and p53 compete for the same coactivator, CBP/p300, effectively inhibiting each other (89–93). On the other hand, it has been shown that p65/p50 induces p53 transcription (94–96) (later shown to require AP-1 and Myc/Max transcription factors [97]).

Also shown in Fig. 7 is the interaction between the NF- κ B and the E2F-Myc nodes. NF- κ B induces transcription of Myc whereas E2F-1 represses the activation of NF- κ B by inhibiting the degradation of I κ B (98) or by binding to the p65 subunit (27), or through some other unknown proteins (99). The influence of NF- κ B on apoptosis and the cell cycle is therefore intertwined with that of the E2F-Myc transcriptional node as discussed in a previous section.

NF- κ B regulates the cell cycle via induction of cyclin D1 (100) and c-Myc (101), factors that promote G1-to-S progression. There is also evidence indicating the presence of NF- κ B binding sites upstream of cyclin D2 (102) and cyclin D3 (103) genes. Studies (104–106) have shown that NF- κ B represses GADD45 ($-\alpha$, $-\gamma$), which in turn promotes cell survival by blocking JNK-induced apoptosis. Mak and Kultz (107) further showed that the various GADD45 isoforms ($-\alpha$, $-\beta$, $-\gamma$) promote early stages of apoptosis but inhibit mitosis. A study by Taylor and Stark (108) also showed that p53 induces G2/M arrest partly through GADD45 proteins.

6. Conclusion

This chapter has presented a perspective for viewing the complexity of intracellular molecular networks associated with a cell's response to growth and death factors in its environment. These responses are coordinated by certain transcription factors which are referred to in this chapter as “control nodes” because each represents a meeting point of signalling pathways (inputs) and pathways (outputs) that diverge to the cell cycle and apoptosis machineries. The complexity of these molecular networks is reduced by subdividing them into modules and by assuming that the module–module interactions in a network are sufficient to understand the essential behaviour of the entire network.

Although only three transcriptional control nodes were discussed in this chapter there are others that can also affect both the apoptosis and cell-cycle programs, including, among others, the Smad family of transcription factors and FOXO (4). The E2F-Myc node (primarily represented by the E2F-1 and c-Myc heterodimeric transcription factors) was discussed above to illustrate transcription factors that potentiate both the initiation of the cell cycle and the apoptosis programs. It may be surprising at first why such a possibility exists at all given that cell cycle and death are diametrically opposite cell fates; but, as discussed above, such a nodal link does provide a mechanism for the organism to carry out the fragile balance between cell proliferation and death (e.g., during its multicellular development). As explicitly analyzed by Aguda and Algar (2) through mathematical modelling, the module–module interactions allow a number of cell fates to be orchestrated sequentially—namely, quiescence, cell cycling, and death—as growth factor signalling intensities increase. The feedback loops in these modular interactions played crucial roles in the behavioural transitions of the system.

The second transcriptional node discussed was p53, a protein of high significance because of its tumor suppressor activity. As summarized in Fig. 5, the core p53-Mdm2-ARF regulatory module is linked to the Akt, p38MAPK, and β -catenin signaling pathways that involve feedback loops. It was pointed out that the mutual antagonism between p53 and Akt is a good candidate for a switching mechanism that toggles between cell death and survival. How this toggle switch is controlled by the associated pathways would be an interesting topic for kinetic modelling.

The NF- κ B family of transcription factors was used as an example of a transcriptional control node that inhibits apoptosis but promotes the cell cycle. However, as discussed

above, it is now known that NF- κ B can also induce expression of proapoptotic genes, and it is still an open question as to how these opposing contributions to apoptosis are sorted out in a given cell. The positive- and negative-feedback loops involved in NF- κ B signaling (*see* Fig. 7) have been the subject of recent mathematical modelling of temporal control of gene expression (109,110).

Lastly, the three transcriptional nodes discussed here interact in various ways as shown in Figs. 2, 4, and 7. It is probable that only a subset of these interactions is realized for any given set of extracellular stimulus. The real challenge is to understand the logic of these interactions, their control, and to predict whether the cell proliferates, survives or die.

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II

MOLECULAR BASIS OF DISEASE THERAPY

Regulation of NF- κ B Function

Target for Drug Development

Daniel Sliva and Rakesh Srivastava

Summary

Nuclear factor- κ B (NF- κ B) is a family of transcription factors instrumental in a variety of physiological as well as pathophysiological conditions. Aberrant activation of NF- κ B is responsible for the overexpression of different genes resulting in unregulated cell proliferation, survival, angiogenesis, cell adhesion, migration, and invasion that had been linked to cancer metastasis. Because the constitutively activated NF- κ B had been demonstrated in a wide variety of cancers, its inhibition is a natural target for the development of new anticancer drugs. The activity of NF- κ B is regulated by proteasome degradation, phosphorylation of NF- κ B, and acetylation/deacetylation of histones. Therefore, compounds specifically suppressing proteasome activity, activities of kinases responsible for the phosphorylation of p65 subunit of NF- κ B, and acetylation/deacetylation associated with NF- κ B activation may be effective in the treatment in a wide variety of cancers.

Key Words: NF- κ B; gene expression; cancer; cell growth; survival; angiogenesis; metastasis.

1. Introduction

In 1986, Sen and Baltimore (1) identified a nuclear factor, which bound to a sequence in the immunoglobulin κ -light-chain enhancer in B-cells stimulated with lipopolysaccharide. Although originally named nuclear factor- κ B (NF- κ B) in accordance with its discovery in B-cells, during the past 20 yr NF- κ B has become a paradigm of transcription factor regulating of a variety of genes in numerous different cells and tissues. Because NF- κ B is essential for immunity, cell proliferation, inflammation, and apoptosis, the activation of NF- κ B has been linked to a variety of physiological as well as pathophysiological conditions and diseases.

NF- κ B family of proteins consists of a group of structurally related and evolutionary conserved subunits that have been identified and cloned in mammalian cells (2). These include Rel (c-Rel), RelA (p65), RelB, NF- κ B1 (p50/p105), and NF- κ B2 (p52/p100). The NF- κ B subunits form homo- or heterodimers through their rel homology domain (RHD), which is also responsible for the DNA binding of NF- κ B and interaction of NF- κ B with I κ B, the family of inhibitory proteins of NF- κ B (3). NF- κ B usually exists in a latent state in the cytoplasm and its activation requires extracellular stimuli leading to the phosphorylation and subsequent proteasome-mediated degradation of inhibitory I κ B proteins (4). Finally, dimeric activated NF- κ B translocates into the nucleus and controls the expression of target genes. However, constitutively active NF- κ B was identified in a variety of cancers and linked to the oncogenesis and tumor resistance (5).

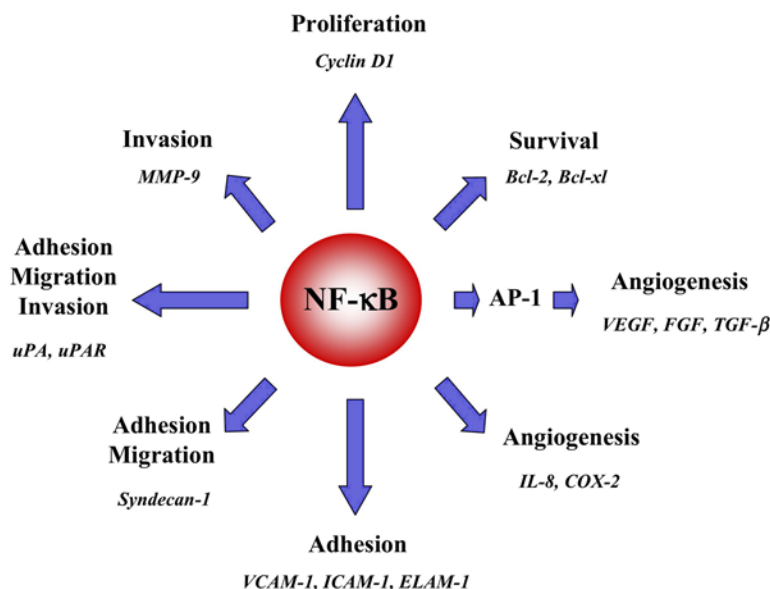


Fig. 1. NF-κB regulated genes involved in cancer growth and metastasis. Expression of genes which are crucial for the normal cell function were also identified in cancer growth and metastasis. These genes are responsible for cell proliferation, cell survival, angiogenesis, cell adhesion, migration, and invasion. However, the function of some gene products can be overlapping.

2. NF-κB and Oncogenesis

The involvement of NF-κB in cancer has been originally suggested because of the homology of mammalian c-Rel with highly oncogenic retroviral v-Rel, which induces aggressive tumors in chicken (6,7). In mammalian cells, NF-κB also regulates expression of more than 150 genes and some of them were linked to cancer initiation, proliferation, angiogenesis, survival, and metastasis (8) (see Fig. 1). Interestingly, NF-κB activation was detected before tumor initiation, therefore connecting aberrant stimulation of expression of NF-κB-regulated genes prior to growth and progression of cancer (9).

2.1. NF-κB and Cell Proliferation

Mitotic cellular division is a crucial feature in the growth of normal as well as cancer cells. The cellular division can be characterized as a cycling process where cells are proceeding from the resting stage (G_0) to DNA synthesis (S) and mitosis (M) stages of cell cycle. During the cell cycle the cells have to go through gap phases G_1 and G_2 , which occur before and after DNA synthesis (S) phase. Cell division is conceived as a passage through a series of checkpoints, with the earliest point late in G_1 when cells become irreversibly committed to DNA synthesis and division (10,11). The checkpoints are controlled by a group of D cyclins, as well as cyclins E and A. Cyclin D1 acts in G_1 and promotes progression through the G_1 -S phase of the cell cycle in mammalian cells. The cyclin-D1 protein is a regulatory subunit of holoenzyme, forming a complex with catalytic subunits of cyclin-dependent kinases (CDKs) cdk4 and cdk6, which phosphorylates and inactivates the retinoblastoma protein pRB

(10,11). The checkpoints of the cell cycle are usually deregulated in oncogenesis, and amplification or overexpression of cyclin D1 have been identified in the development of a subset of human cancers including cancers of breast (12,13), prostate (14), lung (15), colon (16), bladder (17,18), ovary (19), liver (20), pancreas (21), brain (22), and esophagus (23). Furthermore, overexpression of cyclin D1 has been identified in melanomas (24), sarcomas (25), parathyroid adenomas (26), myelomas (27), and myeloid leukemias (28). In addition to the regulation of expression by NF- κ B, the cyclin-D1 regulatory region also contains binding sites for activator protein (AP-1) (c-Jun/c-Fos) (29), STAT5 (30), E2F (31), Sp-1/Sp-3 (31), lymphoid enhancer factor-1 (LEF-1)/ β -catenin (32), and CREB/CREM proteins (33). However, the constitutive activation of NF- κ B (and AP-1) is probably the major factor responsible for the overexpression of cyclin D1 in a variety of cancers. The role of cyclin D1 in tumorigenesis was also confirmed by the overexpression of cyclin D1 in transgenic mice, which resulted in tumors of the mammary gland (34).

2.2. NF- κ B and Apoptosis

Apoptosis is a morphologically and biochemically specific form of cell death during physiological (e.g., embryological development and normal tissue turnover) as well as pathological conditions (including cancer, viral infections, autoimmune diseases, neurodegenerative disorders, and AIDS) (35). In addition to the deregulation of cell proliferation, the suppression of cell death is crucial for the initiation and progression of a variety of cancers (36). Apoptosis can be morphologically recognized by the blebbing of the plasma membrane—condensation of chromatin at the periphery of the nucleus—followed by disintegration of the cell into multiple membrane-enclosed vesicles, and nuclear fragmentation (37). Biochemically, apoptosis can be the result of: (1) the activation of the classical apoptotic pathway through the mitochondrial cytochrome *c* and activation of proteases (caspases); (2) the necrotic programmed cell death mediated by the release of reactive oxygen species; or (3) the release of apoptosis inducing factors leading to the cell death without characteristic nuclear DNA fragmentation (38,39). Caspases activation is controlled by the Bcl-2 family members, which are the key regulators that control release of cytochrome *c* and other apoptosis-promoting factors from mitochondria. Bcl-2 proteins have antiapoptotic as well as proapoptotic functions, and the expression of antiapoptotic Bcl-2 and Bcl-xl was linked to a variety of chemotherapy resistant cancers (40). For example, overexpression of Bcl-2 was associated with apoptosis loss in breast cancers and lymph nodes metastases (41), Bcl-2 is frequently overexpressed in prostate cancer (42), small-cell lung carcinoma (43), colorectal adenomas and carcinomas (44), cancers of bladder (45), kidney (46), and in B-cell chronic lymphocytic leukemia and non-Hodgkin's lymphoma (47). Another antiapoptotic NF- κ B regulated protein Bcl-xl, has been identified in invasive breast cancers (48), and its expression correlated with increasing Gleason score and the presence of prostate cancer metastases (49). In addition, expression of Bcl-xl was associated with the chemoresistance and chemotherapy failure in the treatment of ovarian cancer (50). Bcl-xl overexpression was also identified in lung tumor cell lines (51), colorectal cancers (52), head and neck cancers (53), metastatic malignant melanoma (54), pancreatic cancer (55), and lymphomas (56). Therefore, small molecule

ligands or antisense oligodeoxynucleotides for Bcl-2 and Bcl-xl, were recently suggested in preclinical testing to disrupt survival and chemoresistance properties of Bcl-2 and Bcl-xl in tumor cells (57).

2.3. NF- κ B and Angiogenesis

Angiogenesis, the formation of blood vessels by capillaries sprouting from pre-existing vessels, is important for normal physiological processes such as embryogenesis, growth, and wound healing, as well as pathological processes such as tumor growth and metastasis (58). Angiogenesis is a complex multistep process involving close orchestration of endothelial cells, the extracellular matrix, and angiogenic factors. Although vascular endothelial growth factor (VEGF) was originally the only growth factor proven to be specific for angiogenesis, other molecules have been implicated as positive regulators of angiogenesis, including fibroblast growth factor (FGF), transforming growth factor (TGF)- β , interleukin (IL)-8, cyclooxygenase (COX)-2, and others (for a review, *see ref.* 59). Expression of these angiogenic factors can be controlled by NF- κ B indirectly (e.g., VEGF, FGF, TGF- β) or directly by NF- κ B binding to the promoter region of specific genes (e.g., IL-8 and COX-2).

The up-regulation of VEGF expression has been detected in a variety of tumors including breast (60,61), prostate (62,63), lung (64), gastrointestinal tract (65), kidney and bladder (66), brain (67), ovary (68), and thyroid (69). In addition, increased levels of serum concentrations of VEGF were detected in solid tumors (70–72) as well as leukemia and non-Hodgkin's lymphomas (73). Although NF- κ B controls expression of VEGF (74), the VEGF promoter contains binding sites for AP-1, AP-2, Sp-1 (75), hypoxia-inducible factor (HIF)-1 α (76), and STAT3 (77) but not for NF- κ B. As recently demonstrated, NF- κ B modulates the expression of VEGF indirectly through the induction of expression of c-Fos and AP-1 DNA-binding activity (78).

FGF overexpression has been detected in cancers of breast (79,80), prostate (81), lung (82), ovary (83), brain (84), thyroid (85), as well as in melanomas (86) and non-Hodgkin's lymphoma (87). In addition, TGF- β was overexpressed in cancers of breast (88), prostate (89), in lung cancer cells (90), melanomas (91), liver and pancreas tumors (92,93), and Hodgkin's lymphoma (94). Because the promoter region of FGF contains AP1, AP2, and Sp1 sites (95) and the promoter regions of TGF- β have Sp1, AP-1, EGR-1 binding sites (96,97), it is possible that NF- κ B can activate expression of FGF and TGF- β indirectly through the activation of AP-1 (78,98).

The expression of other two factors implicated in angiogenesis, IL-8 and COX-2, is directly controlled by NF- κ B (99,100). In addition, their promoter regions contain binding sites for AP-1 and NF-IL6 for IL-8 (99), and AP-1, NF-IL6, NFAT, and PEA3 for COX-2 (100). Thus, IL-8 overexpression has been detected in cancers of breast (101), prostate (102), lung (103), colon (104), ovary (105), melanoma (106), thyroid (107), brain (108), pancreas (109), leukemia (110), and Hodgkin's disease (111). Although the overexpression of COX-2 was originally described mainly in colorectal cancers (112,113), COX-2 was also linked to the cancer of breast (114,115), prostate (116), lung (117), bladder (118), skin (119), pancreas (120), myeloid leukemia (121), and clinical correlation between the COX-2 expression and prognostic factors in lymphoma patients was recently reported (122).

2.4. NF- κ B and Metastasis

Tumor invasion and metastasis are multifaceted processes involving cell adhesion, cell migration, and proteolytic degradation of tissue barriers (123). Cell adhesion can be mediated through various cellular adhesion molecules, including vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, and endothelial-leukocyte adhesion molecule (ELAM)-1 (or E-selectin), which are regulated by NF- κ B and AP-1 (124–126). Significantly higher levels of VCAM-1 and ICAM-1 were detected in the patients with breast, ovarian, gastrointestinal and myeloma cancers (127), gliomas (128), pancreatic cancer (129), and in patients with Hodgkin's disease (130). VCAM-1 levels were elevated in patients with cancers of prostate (131), colon (131), and lymphoid and myeloid leukaemias (132). ICAM-1 levels were elevated in sera from patients with melanoma (130) and expressed in pulmonary adenocarcinomas (133) and thyroid cancers (134). Elevated levels of ELAM-1 were detected in the cancer of breast, ovary, gastrointestinal (127), prostate (135), lung (136), colon (137), laryngeal carcinoma and oral carcinomas (138), acute leukemia (139), and Hodgkin's disease (140). In addition to the typical cell adhesion molecules (VCAM-1, ICAM-1, and ELAM-1), other proteins were also implicated in cancer metastasis through cell adhesion, cell migration and cell invasion.

The expression of syndecan-1 and syndecan-4 (ryudocan), members of transmembrane heparan and chondroitin sulfate proteoglycans involved in cell–matrix signaling (141), is also controlled by NF- κ B (142,143). Although expression of syndecan-1 inhibited invasion of lymphocytes (144), overexpression of syndecan-4 decreased cell migration of Chinese hamster ovary (CHO) cells (145), and the loss of syndecan expression was linked to tumor progression in a variety of cancers, other studies suggested tumor promoter function of syndecans (146). Thus, increased expression syndecan-1 was detected in breast carcinomas with an aggressive phenotype and poor clinical behavior (147) and in hormone-refractory and metastatic prostate cancers (148). In addition, enhanced syndecan-1 expression has been detected in ovarian (149), pancreatic (150), and gastric cancers (151), and the serum levels of syndecan-1 correlated with increased tumor aggressiveness and poor outcome of lung cancer patients (152). Syndecan-1 was also expressed in chronic lymphocytic leukaemia (153), myeloma (154), and Kaposi sarcoma-associated herpesvirus B-cell lymphomas (155). Interestingly, overexpression of syndecan-4 was only detected in hepatocellular carcinomas and malignant mesotheliomas (156,157). Therefore, the contradictory expression of syndecans in different cancers may reflect tissue and/or tumor stage-specific function, or may reflect the multiple functions of these integrin coreceptors (146).

The urokinase-type plasminogen activator (uPA) is a serine protease that cleaves the extracellular matrix and stimulates the conversion of plasminogen to plasmin (158). Plasmin can mediate cell invasion directly by degrading matrix proteins such as collagen IV, fibronectin, and laminin or indirectly by activating matrix metalloproteinases MMP-2, -3, and -9 and uPA (159–162). Although originally identified for its proteolytic activity, uPA also binds to its receptor uPAR and form complexes with integrin receptors, which in turn modulate signaling pathways responsible for cytoskeleton reorganization resulting in cell adhesion and migration (163). Expression of the uPA gene is controlled through the uPA promoter, which contains functional binding sites for the

transcription factors AP-1, NF- κ B, and PEA3 (164,165), and expression of the uPAR gene is regulated by the transcription factors AP-1, AP-2, NF- κ B, and Sp1 (166). Both uPA and uPAR were identified in a variety of tumors and the correlation between uPA and uPAR overexpression and poor prognosis suggested (167). Therefore, increased levels of uPA and/or uPAR in serum or tumors were identified in patients with breast (168), prostate (169), lung (170), colon (171), ovarian (172), bladder (173), endometrial (174), head and neck cancers (175), melanoma (176), and acute leukemia (177). Finally, inhibition of NF- κ B resulted in the suppression of uPA secretion followed by inhibition of cell adhesion, migration, and invasion of highly metastatic breast cancer cells (178,179).

Matrix metalloproteinases (MMPs) are a family of zinc-containing endopeptidases, which are responsible for the degradation of extracellular matrix during cell invasion in physiological (tissue remodeling during wound healing, embryonic development, and immune cell migration) as well as pathological processes as in cancer metastasis (180). Especially, the expression of MMP-9 (type IV collagenase/gelatinase B), which is controlled by NF- κ B and AP-1 (181), has been correlated with metastasis in several systems. Plasma levels and/or expression of MMP-9 were elevated in cancers of breast (182), prostate (183), non-small cell lung cancer (NSCLC) (184), colon (182), bladder (185), ovary (186), kidney (187), brain (188), melanoma (189), leukemia (190), and lymphoma (191) and the levels of MMP-9 used as a prognostic factor of cancer progression.

3. Regulation of NF- κ B

As summarized above, NF- κ B controls the expression of genes involved in growth, survival, angiogenesis and metastasis in a variety of cancers (Table 1). Therefore, the activity of NF- κ B is a natural target for the inhibition of aberrantly expressed proteins. The “classical pathway” of NF- κ B activation, consisting of proteasome degradation of the natural inhibitor I κ B and subsequent NF- κ B dimerization, nuclear translocation and stimulation of gene transcription, is well recognized (3,4). However, understanding how NF- κ B is regulated will open more possibilities for the targeted modulation/inhibition of its activity.

3.1. Proteasome Degradation

The “classical” NF- κ B activation pathway (for NF- κ B subunits p50 and p65) is triggered by a variety of stimuli through specific receptors resulting in the activation of I κ B-kinase (IKK) complex containing IKK α , IKK β , and IKK γ (see Fig. 2). IKK activated complex in turn phosphorylates I κ B α at Ser32 and Ser36 and is subsequently ubiquitinated and degraded by proteasome (192). Alternative NF- κ B activation pathway (for NF- κ B subunits p52/p100 and RelB) employs NF- κ B-inducing kinase (NIK), which activates an IKK α homodimer. Subsequently, IKK α phosphorylates p100 subunit of NF- κ B, which is ubiquitinated and cleaved into a p52 subunit and forms the active NF- κ B heterodimer with RelB (192). Finally, the third pathway (“atypical”) is independent of IKK, but still requires phosphorylation of I κ B α by DNA damage induced p38-activated casein kinase 2 (CK2) followed by the I κ B α degradation by proteasome (193). The result of the activation of all three pathways is the translocation of NF- κ B (p50/p65 or p52/RelB) complexes into the nucleus, where they activate gene expression. In addition, p65 in p50/p65 and RelB in p52/RelB must be phosphorylated for their optimal transcription activity (194).

Table 1
Overexpression of NF- κ B Regulated Genes in Cancer

	BCA	PrCA	LCA	PaCA	CCA	OCA	LEU
Proliferation							
Cyclin D1	+	+	+	+	+	+	+
Survival							
Bcl-2	+	+	+		+		+
Bcl-xl	+	+	+	+	+	+	
Angiogenesis							
VEGF	+	+	+		+	+	+
FGF	+	+	+			+	
TGF- β	+	+	+	+			
IL-8	+	+	+	+	+	+	+
COX-2	+	+	+	+			+
Adhesion							
VCAM-1	+	+		+	+	+	+
ICAM-1	+		+	+		+	
ELAM-1	+	+	+		+	+	+
Adhesion/Migration							
Syndecan-1	+	+	+	+	+		+
Adhesion/Migration/ Invasion							
uPA	+	+	+		+		+
uPAR	+	+	+		+	+	+
Invasion							
MMP-9	+	+	+		+	+	+

Note: NF- κ B regulated genes that are overexpressed and/or have elevated serum levels elevated in leading cancer types. BCA, breast cancer; PrCA, prostate cancer; LCA, lung cancer; PaCA, pancreatic cancer; CCA, colon cancer; OCA, ovarian cancer; LEU, leukemia. References according to specific cancer may be found in the main text.

As mentioned previously, proteasome degradation of I κ B α is a crucial step in the liberation of NF- κ B and activation of gene transcription. The proteasome complex is a 26S multicatalytic protease composed of a 20S core catalytic complex with 14 different subunits and 19S regulatory complex that recognizes ubiquitinated proteins and regulate the activity of 20S (195). Phosphorylated I κ B α that will be degraded is first marked with ubiquitin chains by three subsequent reactions. Ubiquitin is activated by a ubiquitin-activating enzyme (E1), transferred to a ubiquitin-conjugating enzyme (E2), and finally ubiquitin-protein ligase (E3) transfers ubiquitin to lysine residues 21 and 22 of I κ B α (196). The ubiquitinated I κ B α is then recognized by 19S and degraded by 20S complexes, respectively, resulting in the release of NF- κ B (197). Therefore, specific proteasome inhibitors were developed and screened for their antitumor activity (198). One of them, bortezomib (Velcade), formerly known as PS-341, is the first proteasome inhibitor, which has been recently approved by the Food and Drug Administration for the treatment of newly diagnosed and relapsed/refractory multiple myeloma (199). In addition, bortezomid has also recently shown significant preclinical and clinical activity in various hematologic and nonhematologic malignancies (200–202).

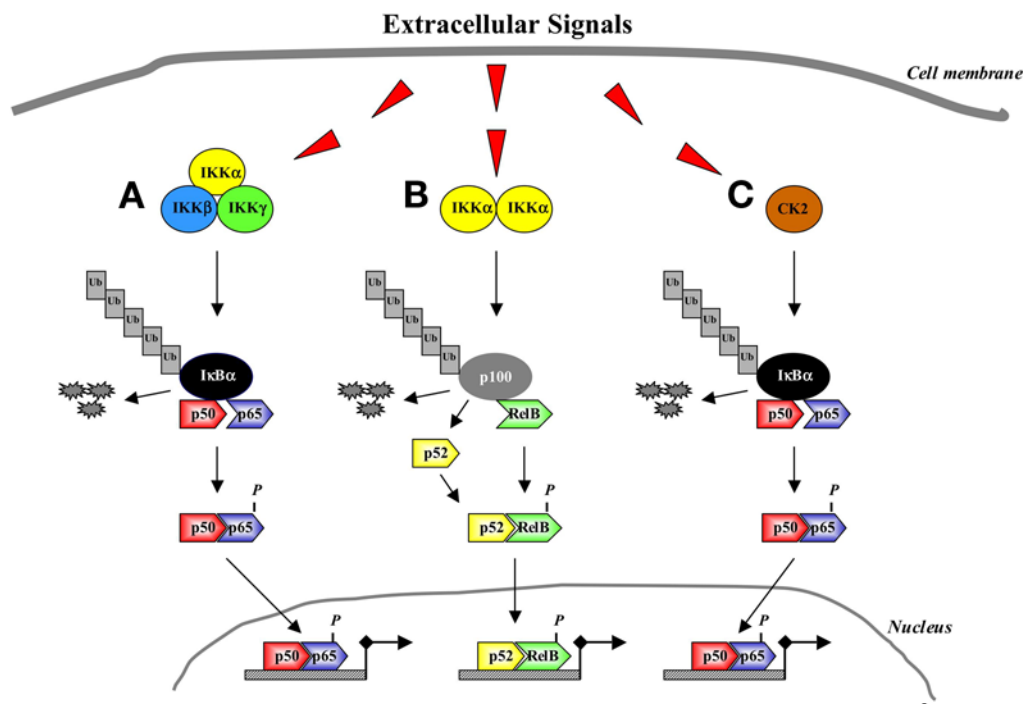


Fig. 2. Mechanism of NF-κB activation by proteasome degradation. (A) The “classical” NF-κB activation pathway through the activation of IKK complex (IKKα, IKKβ, IKKγ) results in the phosphorylation of IκBα and its ubiquitination and degradation leading to the liberation of NF-κB (p50 and p65) and NF-κB nuclear translocation. (B) The “alternative” NF-κB activation pathway through the activation of IKKα homodimers results in the phosphorylation of p100 protein and its proteolysis leading to the release of p52 which forms complex with RelB and translocates into nucleus. (C) The “atypical” NF-κB activation pathway through the activation of CK2 results in the degradation of IκBα and the liberation of NF-κB (p50 and p65) and NF-κB nuclear translocation.

3.2. Phosphorylation of NF-κB

In addition to the phosphorylation and subsequent degradation of IκBα, direct phosphorylation of NF-κB is also required for its optimal DNA-binding and transcription activation. Different protein kinases were identified to be necessary for the phosphorylation of specific sites in the NF-κB subunit p65 (see Fig. 3). Therefore, protein-kinase-A catalytic subunit (PKAc) phosphorylates p65 at Ser276 in the cytoplasm (203), whereas mitogen- and stress-activated protein kinase-1 (MSKM1) phosphorylates the same site of p65 in the nucleus (204). Protein kinase C-ζ (PKC-ζ) induces the activity of NF-κB through the phosphorylation of p65 at Ser311 (205) and casein kinase 2 (CK2) (206), IKKα (207), and Akt (208) phosphorylates p65 at Ser529. Interestingly, Ser536 in p65 was the target for the phosphorylation of a variety of kinases including IKKα (207,209), IKKβ (209), IKKε (209), TRAF family member-associated (TANK)-binding kinase 1 (TBK1) (209), serine/threonine kinases Akt (208), ribosomal S6 kinase 1 (RSK1) (210), and Bruton’s tyrosine kinase (Btk) (211). In addition, glycogen synthase kinase (GSK)-3β phosphorylated p65 on COOH-terminal transactivation domain, which contains four potential GSK-3 phosphorylation sites within amino acids 354-441 (212).

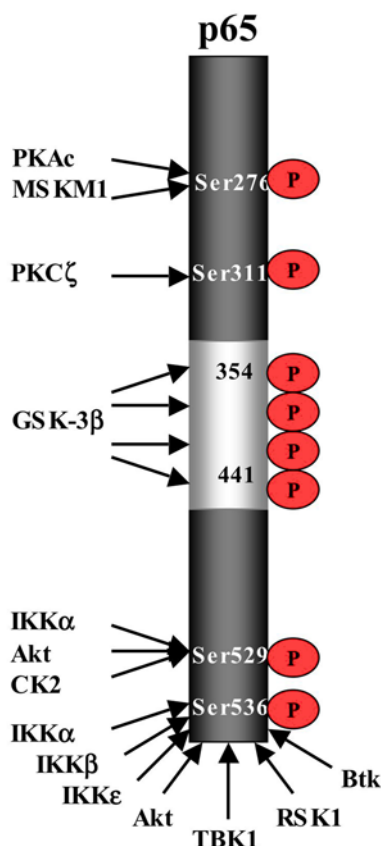


Fig. 3. Kinases phosphorylating p65 subunit of NF- κ B. Direct phosphorylation of NF- κ B enhances DNA-binding and transcription activation. Akt, serine/threonine kinase; Btk, Bruton's tyrosine kinase; CK2, casein kinase 2; GSK-3 β , glycogen synthase kinase-3 β ; IKK, I- κ B kinase; MSKM1, mitogen- and stress-activated protein kinase-1; PKAc, protein kinase A catalytic subunit; PKC- ζ , protein kinase C- ζ ; RSK1, ribosomal S6 kinase; TBK1, TRAF family member-associated (TANK)-binding kinase 1.

As demonstrated previously, different kinases are necessary for the phosphorylation of p65 and NF- κ B activation. However, these data were obtained in different cell lines and with different biological stimuli. In addition, some of the phosphorylation sites might not be necessary for the full NF- κ B activity in a particular system. Nevertheless, the kinases responsible for the phosphorylation of p65 are promising targets for the inhibition of aberrantly active NF- κ B in cancer.

3.3. Acetylation and Deacetylation

Gene expression is a tightly regulated process requiring the fine-tuning of the transcription machinery through the establishment of an active transcription complex (213). In its nonactive state, eukaryotic DNA is condensed into chromatin through the coiling of DNA on the surface of nucleosome resulting in the suppression of gene expression (214). Transcription coactivators are able to uncoil the DNA from the nucleosome core (containing histone proteins H2A, H2B, H3 and H4) by acetylation of histones, therefore

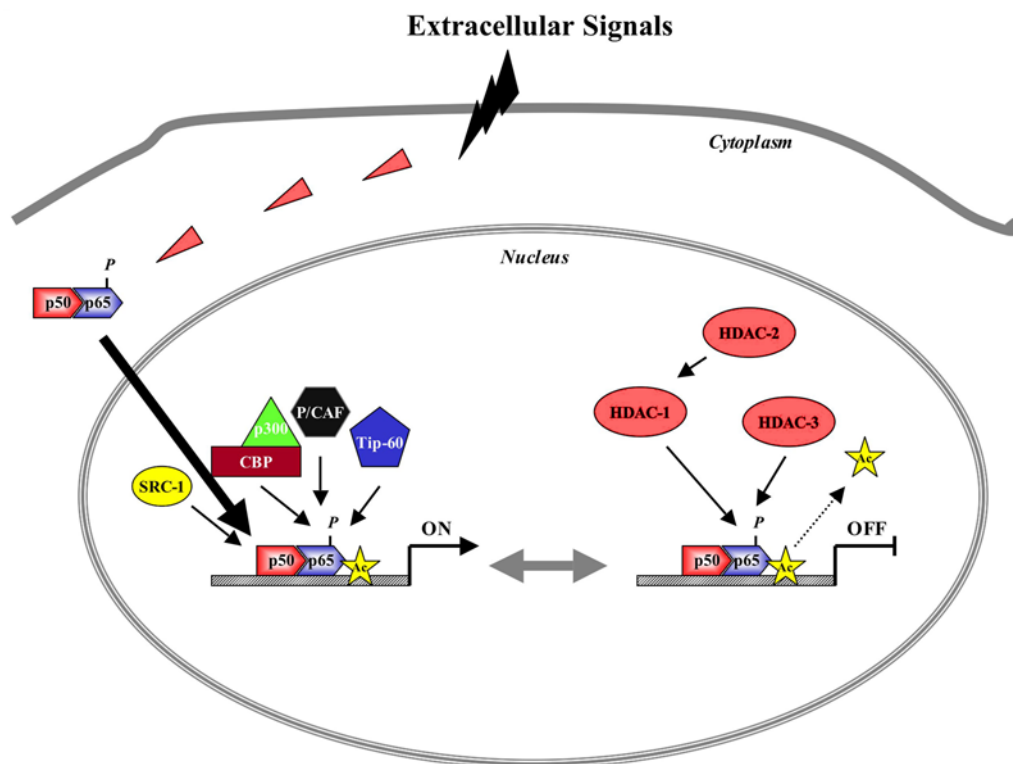


Fig. 4. Regulation of gene expression by acetylation/deacetylation. Direct interaction between HATs CBP/p300, p/CAF, Tip60, or SRC-1 with NF- κ B results in histone acetylation and activation of gene expression. The interaction between HDACs and NF- κ B results in histone deacetylation and suppression of gene expression. CBP/p300, cyclic AMP response element-CREB binding protein-CBP and p300; p/CAF, CBP-associated factor; SRC-1, steroid receptor coactivator-1.

opening and increasing the accessibility of DNA for the binding of transcription factors (215). Because the histone acetylation is a reversible process, the regulation of gene transcription is controlled by coactivators—histone acetylases (HATs) and by corepressors—histone deacetylases (HDACs) (see Fig. 4).

Not surprisingly, the activation of transcription by NF- κ B is regulated by acetylation and deacetylation. Therefore, histone acetylase CBP (cyclic AMP response element-CREB binding protein-CBP) and p300 (CBP/p300) (216), steroid receptor coactivator-1 (SRC-1) (217), CBP-associated factor (p/CAF) (218), and Tip60 (219) potentiated the activation of NF- κ B. Interestingly, phosphorylation of p65 at Ser276 enhanced the interaction of p65 with p300/CBP and stimulated the transcription activity of NF- κ B (220). However, NF- κ B-induced activity by SRC-1 was the result of the interaction of p50 subunit of NF- κ B with SRC-1 (218).

NF- κ B-dependent gene expression was repressed by histone deacetylases HDAC-1, HDAC-2 (221), and HDAC-3 (222). Although HDAC1 repressed NF- κ B activity directly through its interaction with p65, HDAC2 activity on NF- κ B was mediated

indirectly through its association with HDAC1 (221). As mentioned above, phosphorylation of p65 at Ser276 stimulated the transcription activity of NF- κ B through p300/CBP interaction (220) and the same phosphorylation at Ser276 decreased the interaction of HDAC-1 with p65 (223), suggesting that specific phosphorylation of p65 can determine if the activity of NF- κ B will be regulated by coactivators or corepressors.

Although the importance of acetylation/deacetylation of histones was originally described for the activation of transcription mediated by NF- κ B, HATs and HDACs can also directly acetylate/deacetylate p50 and p65 subunits of NF- κ B (224). Therefore, enhanced acetylation of p50 correlates with increased expression of COX-2 (225) and acetylated p65 was reversibly deacetylated by HDAC3, suggesting that HDAC3 can be an intranuclear molecular switch that controls the duration of the transcriptional response (222,226). However, the transcription regulation through NF- κ B is more complicated because acetylation of p65 can activate as well as suppress NF- κ B-dependent gene expression (222,227). Therefore, acetylation of p65 at lysines 218, 221, and 310 activates NF- κ B by inhibiting I κ B α binding to p65. Conversely, acetylation of p65 at lysines 122 and 123 suppresses NF- κ B-dependent gene transcription by reducing DNA-binding to NF- κ B (227). Even though the exact molecular mechanism of acetylation/deacetylation-dependent transcriptional regulation is not fully elucidated, aberrant transcriptional repression by HDAC activity was associated with neoplastic transformation and HDAC inhibitors are considered as a promising new class of cancer therapeutic agents (228).

However, HDAC inhibitor trichostatin A (TSA) demonstrated quite the opposite effects on the expression of NF- κ B-regulated genes, which are involved in cancer proliferation, survival, and metastasis. Thus, TSA increased both basal and TNF-induced expression of IL-8 (221), and TSA stimulated expression of Bcl-xl, MMP-9 and IL-8 in lung cancer cells (229). In contrast, TSA down-regulated the expression of cyclin D1 by the inhibition of DNA binding of NF- κ B/p65 independently of the acetylation status of p65 (230). In addition, the inhibition of expression of NF- κ B-regulated cyclin D1, COX-2, MMP-9 and Bcl-2 by indole-3-carbinol (natural compound with antitumor effects that is found in Brassica vegetables) in leukemia cells was associated with decreased acetylation of p65 (231).

4. Summary

Significant progress has been made in our understanding of the mechanism of the activation of NF- κ B during carcinogenesis, but many important aspects remain to be elucidated. Although the activation of NF- κ B is vital for the proper function of the immune system, its aberrant activation results in inflammation and progression of cancer. Because constitutively activated NF- κ B results in overexpression of genes involved in cancer cell proliferation, survival, angiogenesis, invasion, and metastasis, NF- κ B is a characteristic signature for a wide variety of cancers. Thus, the inhibition of NF- κ B is a rational target for the development of new drugs. Because the activity of NF- κ B is regulated through different signaling mechanisms, their specific inhibition could result in the suppression of particular NF- κ B-dependent gene expression in cancer cells. However, this inhibition should be specifically targeted only for cancer cells without affecting the regular immune function.

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5-Fluorouracil

Molecular Mechanisms of Cell Death

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Summary

5-Fluorouracil (5-FU) has been the mainstay of colorectal cancer treatment for over 40 years. However, response rates for 5-FU in advanced colorectal cancer are modest. Although combining 5-FU with the newer chemotherapeutic agents oxaliplatin and irinotecan has improved response rates, new therapeutic strategies are necessary. Understanding the molecular mechanism by which tumors become resistant to 5-FU is needed if drug resistance is to be overcome. Tumor drug resistance is often due to insufficient chemotherapy-induced cell death. In this chapter, we describe the mechanisms of action of 5-FU, focusing on 5-FU-induced cell death. In the future, strategies aimed at increasing the effectiveness of 5-FU may target the cell death and cell survival pathways that are activated by this drug.

Key Words: 5-Fluorouracil; apoptosis; death receptors; growth factor receptors; Bcl-2.

1. Introduction

5-Fluorouracil (FU) is widely used in the treatment of a range of cancers including breast and cancers of the aerodigestive tract, but has had the greatest impact in colorectal cancer. 5-FU-based chemotherapy improves overall and disease-free survival of patients with resected stage III colorectal cancer (*1*). Nonetheless, response rates for 5-FU-based chemotherapy as a first-line treatment for advanced colorectal cancer are only between 10 and 15% (*2*). Combination of 5-FU with newer chemotherapies, such as irinotecan and oxaliplatin, has improved the response rates for advanced colorectal cancer to between 40 and 50% (*3,4*). However, despite these improvements, new therapeutic strategies are urgently needed. Understanding the mechanisms by which 5-FU causes cell death and by which tumors become resistant to 5-FU is an essential step toward overcoming that resistance. This chapter discusses the mechanisms of action of 5-FU and highlights important determinants of drug sensitivity, focusing, in particular, on regulation of apoptosis in response to 5-FU, as we believe that many future therapeutic strategies will be aimed at enhancing the effectiveness of 5-FU by modulating cell death and cell survival pathways.

2. Mechanism of Action of 5-Fluorouracil

5-Fluorouracil (FU) is converted intracellularly to several active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP) (see [Fig. 1](#)). The active metabolites of 5-FU disrupt

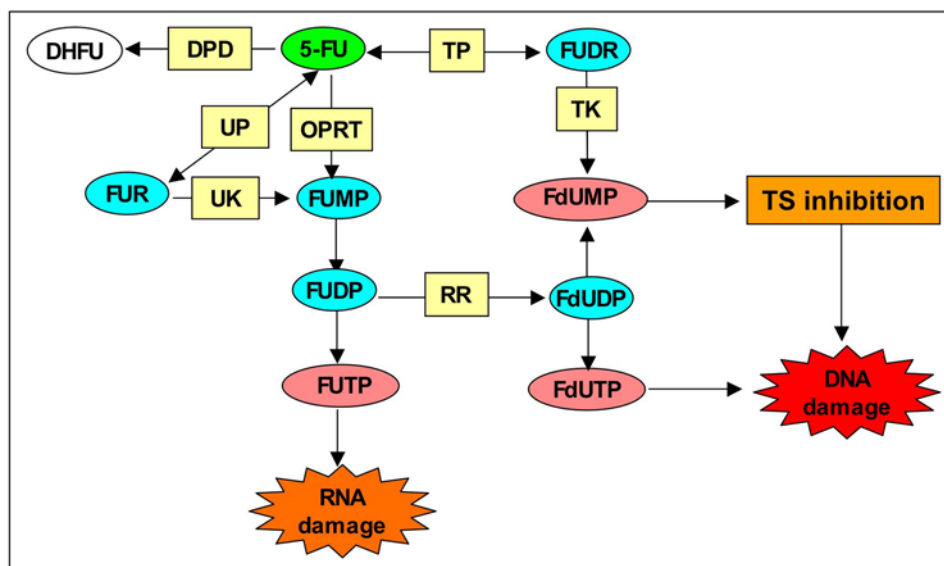


Fig. 1. 5-Fluorouracil (5-FU) is converted to three major active metabolites: (1) fluorodeoxyuridine monophosphate (FdUMP), (2) fluorodeoxyuridine triphosphate (FdUTP), and (3) fluorouridine triphosphate (FUTP). The main mechanism of 5-FU activation is conversion to fluorouridine monophosphate (FUMP) either directly by orotate phosphoribosyl transferase (OPRT), or indirectly via fluorouridine (FUR) through the sequential action of uridine phosphorylase (UP) and uridine kinase (UK). FUMP is then phosphorylated to fluorouridine diphosphate (FUDP), which can be either further phosphorylated to the active metabolite fluorouridine triphosphate (FUTP), or converted to fluorodeoxyuridine diphosphate (FdUDP) by ribonucleotide reductase (RR). In turn, FdUDP can either be phosphorylated or dephosphorylated to generate the active metabolites FdUTP and FdUMP respectively. An alternative activation pathway involves the thymidine phosphorylase catalyzed conversion of 5-FU to fluorodeoxyuridine (FUDR), which is then phosphorylated by thymidine kinase (TK) to the thymidylate synthase (TS) inhibitor, FdUMP. Dihydropyrimidine dehydrogenase (DPD)-mediated conversion of 5-FU to dihydrofluorouracil (DHFU) is the rate-limiting step of 5-FU catabolism in normal and tumor cells.

RNA synthesis (FUTP), inhibit the action of thymidylate synthase (TS)—a nucleotide synthetic enzyme (FdUMP)—and can also be directly misincorporated into DNA (FdUTP). The rate-limiting enzyme in 5-FU catabolism is dihydropyrimidine dehydrogenase (DPD), which converts 5-FU to dihydrofluorouracil (DHFU) (5). Over 80% of administered 5-FU is normally catabolized primarily in the liver, where DPD is abundantly expressed (5).

2.1. TS Inhibition

TS catalyses the reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) with the reduced folate 5,10-methylene-tetrahydrofolate (CH_2THF) as the methyl donor. This reaction provides the sole *de novo* source of thymidylate, which is necessary for DNA replication and repair. TS contains a nucleotide-binding site and a binding site for CH_2THF . The 5-FU metabolite FdUMP binds to the nucleotide-binding site of TS, forming a stable ternary complex with the enzyme and CH_2THF which blocks binding of the normal substrate dUMP, thereby

inhibiting dTMP synthesis (6,7). Inhibition of thymidylate synthesis causes disruption of nucleotide levels that results in DNA damage.

2.2. DNA Damage

The capacity of a cancer cell to repair DNA can determine resistance to chemotherapeutic drugs that induce DNA damage such as 5-FU. The response to DNA damage is either repair or cell death and therefore has a profound effect on tumor chemosensitivity and chemoresistance. Signaling pathways have evolved to arrest the cell cycle following DNA damage to allow more time for DNA repair. Only when repair is incomplete (e.g., when the DNA damage is too extensive) will cells undergo apoptosis. The exact molecular mechanisms that mediate events downstream of TS inhibition have not been fully elucidated. Depletion of dTMP results in subsequent depletion of deoxythymidine triphosphate (dTTP), which induces perturbations in the levels of the other deoxynucleotides (e.g., dATP, dGTP, and dCTP) through various feedback mechanisms (8). Deoxynucleotide pool imbalances (in particular the dATP/dTTP ratio) are thought to severely disrupt DNA synthesis and repair, resulting in lethal DNA damage (9,10). TS inhibition can also result in accumulation of deoxyuridine triphosphate (dUTP), as conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) is blocked (11). Both dUTP and another 5-FU metabolite fluorodeoxyuridine triphosphate (FdUTP) can be misincorporated into DNA. Repair of uracil- and 5-FU-containing DNA is mediated by the base excision repair enzyme uracil-DNA-glycosylase (UDG) (12). However, this repair mechanism is futile in the presence of high (F)dUTP/dTTP ratios, and only results in further false nucleotide incorporation. These futile cycles of misincorporation, excision and repair eventually lead to DNA strand breaks. DNA damage resulting from dUTP misincorporation is highly dependent on the levels of the pyrophosphate dUTPase, which limits intracellular accumulation of dUTP (13,14). Increased dUTPase expression has been associated with resistance to TS inhibitors (13–16).

2.3. RNA Misincorporation

The 5-FU metabolite FUTP is extensively incorporated into RNA, disrupting normal RNA processing and function. Significant correlations between 5-FU misincorporation into RNA and loss of clonogenic potential have been demonstrated in human colon and breast cancer cell lines (17,18). Ribosomes are ribonucleoprotein complexes that play an essential role in the translation of messenger RNA (mRNA) into protein. The RNA component of ribosomes (rRNA) makes up approx 60% of the complex. rRNA is transcribed by RNA polymerase I and is then processed to generate mature rRNA species. 5-FU misincorporation has been found to inhibit the processing of pre-rRNA into mature rRNA (19,20). Furthermore, 5-FU has been shown to disrupt post-transcriptional modification of transfer RNAs (tRNAs), which also play an essential role in mRNA translation (21,22). Small nuclear RNAs (snRNAs) play key roles in the splicing of pre-mRNA (also known as heterogeneous nuclear RNA [hnRNA]) into mature mRNA. 5-FU can disrupt the assembly and activity of snRNA/protein complexes, thereby inhibiting splicing of pre-mRNA (23,24). rRNA, tRNA, and snRNA contain the modified base pseudouridine, and 5-FU has been shown to inhibit the posttranscriptional conversion of uridine to pseudouridine in these RNA species (23,25). These in

vitro studies indicate that 5-FU misincorporation can potentially disrupt many aspects of RNA processing, leading to profound effects on cellular metabolism and viability.

3. Mechanisms of Resistance to 5-Fluorouracil

3.1. Drug Activation/Inactivation

Mechanisms that inactivate drugs can diminish the amount of free drug available to bind to its intracellular target. More than 80% of 5-FU is normally catabolized by dihydropyrimidine dehydrogenase (DPD), primarily in the liver (5). In vitro studies have demonstrated that DPD overexpression in cancer cell lines confers resistance to 5-FU (26). Furthermore, high levels of DPD mRNA expression in colorectal tumors have been shown to correlate with resistance to 5-FU (27), presumably reflecting greater DPD-mediated degradation of 5-FU in these tumors. The activation of 5-FU to its active metabolites is complex, and the levels of 5-FU activating enzymes such as thymidine phosphorylase (TP), uridine phosphorylase (UP), and orotate phosphoribosyl transferase (OPRT) (see Fig. 2) have been associated with 5-FU sensitivity (28–30).

3.2. TS Expression

Alterations in expression levels or mutation of a chemotherapeutic drug target can have a major impact on drug resistance. As mentioned above, the 5-FU metabolite fluorodeoxyuridine monophosphate (FdUMP) is a potent inhibitor of TS, and it is the inhibition of TS that is believed to be the primary anticancer activity of 5-FU (31). Numerous preclinical studies have demonstrated that TS expression is a key determinant of 5-FU sensitivity (32). Furthermore, immunohistochemical and reverse-transcription PCR studies have shown improved response rates to 5-FU-based chemotherapy in patients with low-tumor TS expression (33,34). More recently, genotyping studies have found that patients homozygous for a particular polymorphism in the *TS* promoter (TSER3/TSER3) that increases TS expression are less likely to respond to 5-FU-based chemotherapy than patients who are heterozygous (TSER2/TSER3), or homozygous for the alternative polymorphism (TSER2/TSER2) (35). Collectively, these studies indicate that high-TS expression correlates with increased 5-FU resistance.

Treatment with 5-FU has been shown to acutely induce TS expression in both cell lines and tumors (36,37). This induction of TS seems to be to the result of inhibition of a negative feedback mechanism in which ligand-free TS binds to its own mRNA and inhibits its own translation (38). When stably bound by FdUMP, TS can no longer bind its own mRNA and suppress translation, resulting in increased protein expression. This constitutes a potentially important resistance mechanism, as acute increases in TS would facilitate recovery of enzyme activity. In vitro studies have demonstrated that acute increases in TS expression induce resistance to TS inhibitors such as 5-FU and the antifolate drugs tomudex (TDX) and multitargeted antifolate (MTA, Alimta) (39,40).

3.3. DNA Repair

Meyers et al. found that restoration of hMLH1 in MMR-deficient HCT116 colon cancer cells renders them more sensitive to 5-FU, suggesting that MMR deficient cells are more resistant to 5-FU (41). However, the MSI phenotype has been associated with excellent survival in colorectal cancer patients receiving adjuvant 5-FU-based chemotherapy (42). These apparently contradictory findings may be to the result of intrinsic biological

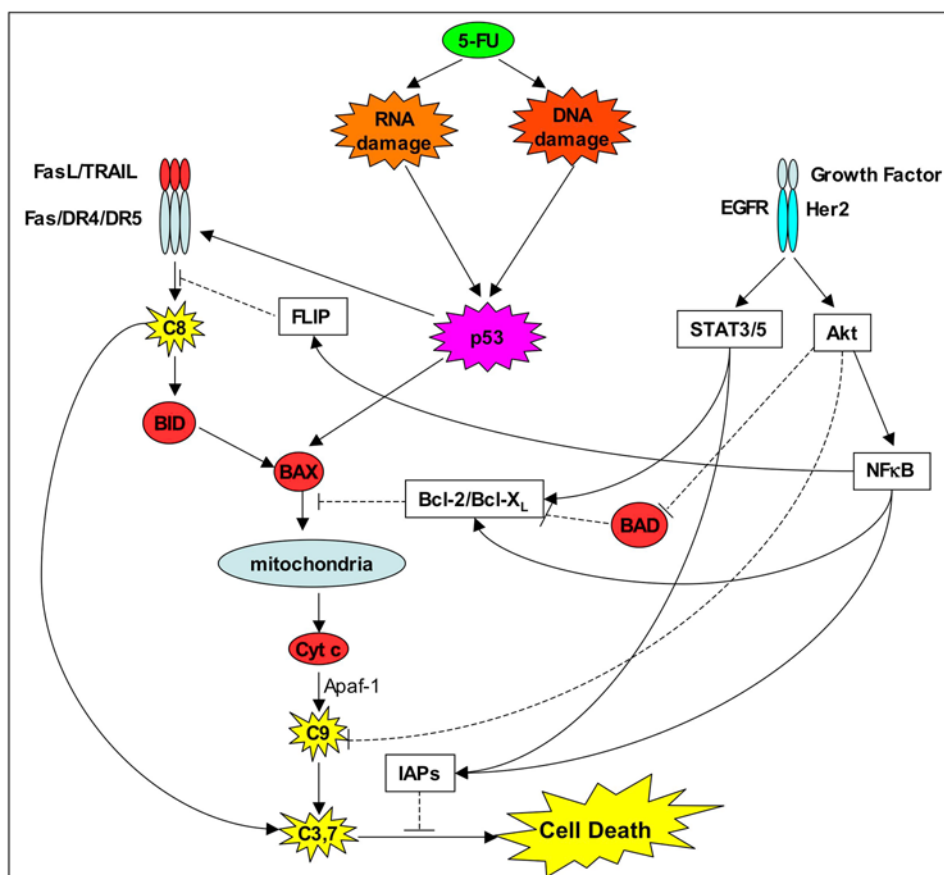


Fig. 2. Interactions between proapoptotic and prosurvival signal transduction pathways that may play important roles in determining response to 5-FU. *Abbr:* Apaf-1, apoptotic protease activating factor; C3, caspase 3; C7, caspase 7; C8, caspase 8; C9, caspase 9; cyt c, cytochrome *c*.

differences between MSI-positive and MSI-negative colorectal tumors, with MSI-positive (MMR deficient) tumors being less aggressive. For example, it has been suggested that MSI may lead to an increased anti-tumor immune response (43). In addition, it has been demonstrated that the majority of MSI-positive tumors express wild type p53 (44), which is an important determinant of 5-FU sensitivity (discussed later).

3.4. *p53*

As already mentioned, there is a critical balance between cell-cycle arrest (i.e., promoting DNA repair and survival) and cell death following chemotherapy. The tumor suppressor protein p53 plays a central role in regulation of cell-cycle arrest and cell death (45). The gene encoding p53, *TP53*, is the most frequently mutated gene in human cancers, with about 50% of all tumors estimated to carry a mutation (46). DNA damage results in activation of upstream kinases such as ATM (Ataxia-telangiectasia mutated), ATR (ATM and Rad-3 related), and DNA-PK (DNA-dependent protein kinase), which can directly or indirectly activate p53 (47). Phosphorylation of p53 by upstream kinases inhibits its negative regulation by MDM-2, which targets p53 for ubiquitin-mediated

degradation (47). The role of p53 in determining cell fate following DNA damage has been attributed to its role as a transcription factor. p53 transcriptionally up-regulates genes such as those encoding p21^{WAF-1/CIP-1} and GADD45, which induce cell-cycle arrest in response to DNA damage (48,49). However, depending on the cellular context, p53 can trigger elimination of the damaged cells by promoting apoptosis through the up-regulation of proapoptotic genes such as *Bax*, *NOXA*, *TRAIL-R2 (DR5)*, and *Fas (CD95/Apo-1)* (50–53). Both p53-induced cell-cycle arrest and apoptosis act to maintain genomic integrity and prevent damaged DNA being passed on to daughter cells. In addition, we have demonstrated that p53 and p53-target genes are activated in response to RNA-directed 5-FU cytotoxicity (40).

A number of experimental reports have indicated that lack of functional p53 contributes to drug resistance, and this has been attributed to an inability to undergo p53-mediated apoptosis. For example, in vitro studies have reported that loss of p53 function reduces chemosensitivity to 5-FU (40,54). Furthermore, a number of clinical studies have found that p53 overexpression (a surrogate marker for p53 mutation) correlated with resistance to 5-FU-based chemotherapy (42,55,56), although other studies failed to find a correlation (57). Such conflicting findings may be due at least in part to the fact that p53 overexpression does not actually reflect *TP53* mutation in as many as 30 to 40% of cases (58). In addition, gain-of-function p53 mutations have been described that actively contribute to transformation and drug resistance (59). Indeed, a recent study has suggested that certain p53 mutants may increase dUTPase expression, resulting in 5-FU resistance (15). So, 5-FU chemosensitivity may be dependent on the particular *TP53* genotype.

4. Induction of Apoptosis

The ultimate goal of cytotoxic chemotherapies is to induce cell death in tumor cells. The onset of apoptosis is regulated by multiple intra- and extracellular signals, amplification of these signals by second messengers, and activation of the effectors of apoptosis—the caspases. There are two main pathways for activation of caspases: the intrinsic pathway regulated by Bcl-2 proteins and the extrinsic pathway regulated by members of the tumor necrosis factor (TNF) receptor superfamily (60). Activation of proximal caspases in these pathways leads to activation of downstream effector caspases, most importantly caspase 3 and 7. These executioner caspases cleave a cassette of cellular substrates to bring about the morphological and biochemical changes that characterize apoptosis, including chromatin condensation and nuclear fragmentation, membrane blebbing, and cell shrinkage (60). Eventually, the cell breaks into small membrane-bound fragments (apoptotic bodies) that are cleared by phagocytosis without causing an inflammatory response.

Activation of the intrinsic apoptotic pathway is regulated by the Bcl-2 family of proteins. Although these proteins share some homology, some Bcl-2 family members, such as *Bax* and *Bid*, promote apoptosis whereas others, such as *Bcl-X_L* and *Bcl-2* itself, are antiapoptotic (60,61). Not surprisingly, the role of Bcl-2 family members in regulating response to chemotherapy has been extensively studied. Several in vitro studies have demonstrated that antisense targeting of *Bcl-2* or *Bcl-X_L* sensitizes cancer cells to 5-FU (62–64), whereas overexpression of *Bax* has been found to sensitize cancer cells to 5-FU-induced apoptosis (65). It has also been demonstrated that loss of *Bax* expression decreases sensitivity to 5-FU (66). Furthermore, Violette et al. found that high levels of

Bcl-2 and Bcl-X_L coupled to low levels of Bax correlated with resistance to 5-FU in colon cancer cell lines (67). In addition, Bid null mouse embryonic fibroblasts were found to be more resistant to 5-FU, and Bid overexpression sensitized hepatocellular carcinoma cells to 5-FU (68,69).

Oblimersen (G3139, Genasense) is an antisense oligonucleotide designed to specifically downregulate Bcl-2 that has been shown to synergize with cytotoxic drugs in pre-clinical models (70). Various clinical trials are currently evaluating the antitumor potential of combining oblimersen with chemotherapy. In terms of the clinical predictive value of Bcl-2 proteins, several studies have shown no correlation between Bcl-2 expression and response to 5-FU-containing chemotherapeutic regimens (71,72). Similarly, Paradiso et al. found no correlation between Bax expression and response to 5-FU-based chemotherapy in patients with advanced colorectal cancer, whereas Sjostrom et al. found no correlation between response to 5-FU-based chemotherapy and Bcl-2, Bcl-X_L, and Bax expression in advanced breast cancers (73,74). So, although Bcl-2 family members undoubtedly play important roles during chemotherapy-induced apoptosis, their usefulness as molecular predictive markers of response appears to be limited.

The extrinsic apoptotic pathway is regulated by cell surface “death receptors” of the TNF-receptor family. These include Fas (CD95/APO-1), DR4 (TNF-related apoptosis-inducing ligand receptor 1, TRAIL-R1), and DR5 (TRAIL-R2). Houghton et al. demonstrated that apoptosis of colon cancer cells in response to thymidylate deficiency was mediated via the Fas pathway, suggesting an important role for this apoptotic pathway following 5-FU-mediated inhibition of TS (75). Furthermore, in vitro studies have shown that targeting death receptors with recombinant death ligands or agonistic antibodies can enhance 5-FU-induced apoptosis (51,76–78). Efficient induction of Fas-mediated apoptosis in response to agonistic Fas antibodies and recombinant FasL has been demonstrated in vivo; however, systemic treatment with Fas-targeted agents has been shown to cause severe liver damage (79). Most preclinical studies are now focused on local administration of rFasL, or the use of FasL-expressing vectors as gene therapy (79). However, Ichikawa et al. have successfully developed a nonhepatotoxic agonistic Fas antibody (80), suggesting that it is possible to develop less toxic Fas antibodies.

A clinical study by Backus et al. found that 5-FU treatment up-regulated Fas expression in colorectal tumors (81), suggesting that Fas is a clinically important mediator of response to chemotherapy. In several cancers, down-regulation of Fas and up-regulation of FasL have been demonstrated to occur during disease progression (82–85). It has been postulated that tumor FasL induces apoptosis of Fas-sensitive immune effector cells, thereby inhibiting the antitumor immune response (86). A recent study found that Fas-negative/FasL-positive breast cancer tumors had significantly shorter DFS and OS following adjuvant chemotherapy (87). However, a number of clinical studies have failed to show any relationship between Fas/FasL expression and response to 5-FU-based chemotherapy (74,88).

Targeting the TNF-related apoptosis-inducing ligand (TRAIL) receptors may prove to be a more promising therapeutic approach than targeting Fas, as TRAIL has been shown to exert marked anticancer activity without systemic toxicity in mice (89). Wang et al., recently found that RNAi-mediated silencing of DR5 increased resistance to 5-FU in colon cancer cells (90). Furthermore, several in vitro studies have demonstrated synergy between rTRAIL and 5-FU (and other chemotherapies) in a number of different cancers (91–93). Furthermore, a study by Naka et al. using fresh surgical specimens taken from

human colon tumors implanted in severe combined immunodeficiency (SCID) mice found that combining rTRAIL with 5-FU (or irinotecan) produced a greatly enhanced antitumor effect as compared with either agent alone (94). Clinical trials are currently examining rTRAIL and TRAIL receptor-targeted antibodies as anticancer agents.

Apoptosis mediated by both Fas and DR4/DR5 can be inhibited by cytoplasmic factors, most notably c-FLIP (a FADD-like interleukin-1 β -converting enzyme-inhibitory protein), which binds to the death-induced signal complex (DISC) and inhibits caspase 8 activation (95). c-FLIP overexpression has been found to inhibit death ligand-induced apoptosis in a number of in vitro studies (96–98). Furthermore, Ganten et al. identified down-regulation of c-FLIP as the mechanism of 5-FU-mediated sensitization to rTRAIL in hepatocellular carcinoma cells (99). Recently, we have found that RNAi targeting of c-FLIP dramatically sensitized a panel of colon cancer cell lines to 5-FU (and irinotecan and oxaliplatin) in the absence of cotreatment with any death ligand, suggesting that c-FLIP plays an important role in regulating colon cancer cell chemosensitivity (99a). Interestingly, c-FLIP has been found to be overexpressed in a high percentage of colonic and gastric carcinomas (100,101), however the significance of c-FLIP overexpression for clinical drug resistance has not yet been studied.

Death receptor-mediated apoptosis can also be inhibited by decoy receptors: decoy receptor 3 (DcR3) in the case of Fas, and DcR1 and DcR2 in the case of the TRAIL receptors (89). These decoy receptors bind to FasL/TRAIL, but lack the intracellular domains necessary for DISC formation and therefore inhibit death receptor-mediated apoptosis. A recent study in stage II and III colorectal cancer found that the *DcR3* gene was amplified in 63% of cases and that the DcR3 protein was overexpressed in 73% of cases (102). Although, adjuvant chemotherapy was found to be significantly more beneficial in patients with normal *DcR3* gene copy number in this study, DcR3 protein expression was not associated with the effectiveness of adjuvant 5-FU-based chemotherapy. So, the clinical significance of decoy receptor expression for drug resistance has yet to be demonstrated; however, decoy receptors may prove to be important clinical determinants of sensitivity to agents targeted directly against death receptors, such as rTRAIL, which may eventually be used in combination with 5-FU.

5. Prosurvival Signaling

Protein tyrosine kinases (PTKs) can have an important impact on drug resistance through their regulation of antiapoptotic signal transduction pathways (103). Overexpression and oncogenic mutations of many PTKs have been described in human cancers (103). Some of the best-characterized PTKs are the epidermal growth factor receptor (EGFR) family, which are comprised of EGFR (ErbB1, Her1), Her2 (ErbB2, Neu), Her3 (ErbB3), and Her4 (ErbB4) (104,105). Binding of growth factors such as epidermal growth factor (EGF), transforming growth factor- α (TGF- α), and heregulins result in homo- and heterodimerisation of EGFR, Her3, and Her4, with the preferred binding partner being Her2, for which there is no known ligand (105). Receptor dimerisation leads to cross autophosphorylation of key tyrosine residues in the receptor cytoplasmic domain, which creates docking sites for downstream signal transducers. Antiapoptotic downstream signals activated by these receptor tyrosine kinases include the phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B, PKB) pathway and the signal transducers and activators of transcription (STAT) pathway, specifically STAT3

and STAT5 ([103,104](#)). Numerous reports have described EGFR overexpression or mutation in various human cancers, indicating an important role for this receptor in cancer ([103,106](#)).

EGFR and Her2 have long been recognized as potential drug targets, and specific inhibitors of these receptors have entered the clinical arena ([106,107](#)). The Her2 inhibitor herceptin (trastuzumab) is used to treat the 20 to 40% of breast cancers that are Her2-positive ([108](#)). The EGFR tyrosine kinase inhibitors gefitinib (Iressa, ZD1839) is currently being tested in clinical trials in a number of solid tumors and is approved for use in the treatment of advanced non-small cell lung cancer ([107](#)). Cetuximab (C225), another EGFR inhibitor, is in phase III trials in combination with chemotherapy in advanced colorectal cancer ([107,109](#)). In vitro and xenograft studies have found that gefitinib enhances the cytotoxic effects of a variety of chemotherapies including 5-FU ([110–113](#)). We have found that 5-FU treatment enhances phosphorylation on EGFR tyrosine residue 1068 (a surrogate marker for EGFR activation) in a panel of colorectal cancer cell lines ([113a](#)). Furthermore, this up-regulation appears to be the molecular basis of the synergy between gefitinib and 5-FU in these cell lines.

STAT proteins transmit cytoplasmic signals from cytokine and growth factor receptors to the nucleus, where they activate transcription of a diverse set of target genes ([114](#)). Persistent activation of STATs, in particular STAT3 and STAT5, has been demonstrated in a large number of cancers, and this often occurs as a result of constitutive activation of an upstream tyrosine kinase ([114](#)). Phosphorylated STAT proteins form activated dimers that translocate to the nucleus and up-regulate target gene transcription. STAT3 and STAT5 have been shown to regulate Bcl-X_L expression and apoptosis in a wide range of tumor cells ([115–120](#)). A study by Real et al. demonstrated that STAT3-dependent overexpression of Bcl-2 inhibited chemotherapy-induced apoptosis in breast cancer cells ([121](#)), whereas Masuda et al. demonstrated that inhibition of STAT3 enhanced the sensitivity of head and neck cancer cells to 5-FU ([122](#)). Given its antiapoptotic activity and the frequency of its activation in cancer, STAT3 is a particularly attractive therapeutic target.

The transcription factor nuclear factor- κ B (NF- κ B) is a key regulator of oncogenesis through its promotion of proliferation and inhibition of apoptosis ([123](#)). NF- κ B exerts its antiapoptotic effects by up-regulating a number of antiapoptotic proteins, including inhibitors of apoptosis proteins (IAPs), TNF-receptor associated factors (TRAFs), c-FLIP, Bcl-2, Bfl-1 (A1), and Bcl-X_L ([123](#)). NF- κ B has been connected with multiple pathways involved in oncogenesis, including cell-cycle regulation and apoptosis ([124,125](#)). Chuang et al. demonstrated that a wide range of cytotoxic drugs including 5-FU activated NF- κ B in a panel of cancer cell lines ([126](#)). This suggests that NF- κ B activation is a general feature of cancer cell response to chemotherapy. Whether constitutively or inducibly activated, NF- κ B appears to be a critical determinant of drug resistance, with NF- κ B activation blunting the ability of chemotherapy to induce cell death ([125](#)). Increased NF- κ B activity in patients with oesophageal cancer has been correlated with reduced response to neoadjuvant 5-FU-based chemotherapy and radiation therapy ([127](#)). Inhibiting NF- κ B signaling may prove to be an effective strategy to enhance 5-FU-induced apoptosis in a range of cancers. NF- κ B is believed to be a major target for proteasome inhibitors, as proteasome inhibition prevents degradation of the NF- κ B inhibitor I κ B ([128,129](#)). Clinical trials with proteasome inhibitors such as bortezomib are underway.

6. Conclusion

In the past, attempts to modulate 5-FU cytotoxicity have focused primarily on increasing activation, decreasing degradation, and enhancing TS inhibition (32). The development of new proteomic and genomic technologies now enables characterization of the downstream signaling pathways involved in regulating tumor cell response to chemotherapy more so than ever before. This will facilitate the future development of rational combined chemotherapy regimens designed to maximize drug activity. Newer therapies that target apoptotic pathways are likely to be used increasingly in combination with cytotoxic drugs to enhance chemotherapy activity. In the future, the ability to predict response to cytotoxic drugs and to modulate this response with targeted therapies will permit selection of the best combined treatment for an individual patient.

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Apoptosis-Inducing Cellular Vehicles for Cancer Gene Therapy

Endothelial and Neural Progenitors

Gergely Jarmy,* Jiwu Wei,* Klaus-Michael Debatin, and Christian Beltinger

Summary

Endothelial progenitor cells (EPCs) and neural progenitor cells (NPCs) are promising for cancer therapy because they specifically target tumors. They have the capacity to home to, invade, migrate within, and incorporate into tumor structures. They are easily expanded and can be armed with therapeutic payloads protected within the progenitor cells. Once in the tumor, armed progenitors can be triggered to induce apoptosis in surrounding tumor cells. Pro- and antiapoptotic mechanisms are pivotal to effectively kill tumor cells while simultaneously protecting the cellular vehicles from premature demise. Increasing the ratio of tumor cell apoptosis to progenitor apoptosis will be crucial among other efforts to enhance the efficacy of endothelial and neural progenitor cells to a level sufficient for clinical application.

Key Words: Apoptosis; endothelial progenitor cells; neural progenitor cells; cellular vehicle; cancer; gene therapy.

1. Cellular Vehicles for Gene Therapy of Tumors

Conventional tumor therapy by surgery, chemotherapy, and radiation often fails or is associated with severe side effects. Improved therapies are therefore needed. This may be achieved by improved targeting, that is, by confining the therapy-induced cytotoxicity to the tumor cells. Recent developments in tumor targeting have led to small molecules, such as receptor tyrosine kinase (TK) inhibitors, aimed at signaling pathways pivotal for tumors, to antibodies targeted at surface molecules, and to targeted gene therapy. However, the degree of targeting is often inefficient: the targets of small molecules may not be as tumor-specific as required and large antibodies may not penetrate the depth of a tumor mass. The success of gene therapy for treatment of cancer, in particular, depends on delivering genes specifically to tumor cells. For this, both viral and nonviral vectors have been used. Despite significant advances, gene therapy approaches using these vectors have been hampered by imprecise tumor targeting leading to unexpected side effects, low-viral titers, rapid degradation by the immune system, nonspecific adhesion, poor transduction efficiency, and low-level transgene expression. Moreover, these vectors have been mostly administered loco-regionally by direct intratumoral injection. Because metastases or inaccessible tumors account for a significant proportion of morbidity and mortality in cancer patients, there is a need for vectors that can be

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administered systemically to target these tumor manifestations. To overcome the limitations of viral vectors, mammalian cells have been proposed as potential gene delivery vehicles. In principle, cellular vehicles offer several advantages. They may be able to home to tumors and mediate tumor cell death while, ideally, being protected against apoptosis induced by their therapeutic payload. If chosen appropriately, they should not evoke an immune response. Furthermore, some cell types intended as vehicles may be readily available, easy to isolate noninvasively, expandable *ex vivo*, and receptive to genetic manipulation. Not surprisingly, a variety of cellular vehicles have been investigated. In a pioneering clinical study genetically marked autologous tumor-infiltrating lymphocytes, expanded and activated *in vitro*, were used as vehicles for tumor-specific immunotherapy (1). Other cell types, including fibroblasts (3–6), macrophages (7), endothelial cells (ECs) (8), natural killer (NK) cells, dendritic cells, and autologous or allogeneic tumor cells (9) have been evaluated as cellular vehicles for tumor gene therapy. However, modest tumor-specific homing, limited *ex vivo* expandability, difficult genetic manipulation, or poor tolerance have hindered clinical translation of these vehicles. By virtue of their intrinsic properties, adult and embryonic stem and progenitor cells promise to overcome these problems. This chapter focuses on two well-characterized progenitors, endothelial cell (EPC) and neural progenitor cells (NPC) as potential cellular vehicles to target two of the most therapy-recalcitrant cancer manifestations: metastases and brain tumors.

2. Endothelial Progenitors for Systemic Apoptosis-Inducing Cancer Gene Therapy

2.1. The Physiological Role of Endothelial Progenitors—Vasculogenesis and Angiogenesis

During development, the vasculature is formed by vasculogenesis, that is the *in situ* differentiation of ECs from their precursors and their subsequent organization into a primary capillary network. Vasculogenesis during development requires EPCs that are derived from a common precursor of both the hematopoietic system and the vascular system called the hemangioblast (10). The crucial role of vascular endothelial growth factor receptor (VEGFR)2 and VEGF in embryonic vasculogenesis is shown by the phenotype of both VEGFR2 and VEGF knock-out mice who die *in utero* as a result of lack of endothelial and blood cells (11,12). In adult life new vessels are formed during wound healing, the endometrial cycle and the growth of tumors. This postnatal vessel formation has for a long time been thought to occur exclusively by sprouting from existing vessels and was termed angiogenesis. Recent work has shown that adult EPCs are recruited from the bone marrow to form new vessels in adults during both physiological and pathological conditions (13,14). Adult EPCs have been characterized as highly proliferative cells, having properties similar to those of embryonic angioblasts. In response to angiogenic stimuli they are mobilized from the bone marrow and after differentiation into mature ECs they functionally incorporate into vessels. These findings have suggested that vasculogenesis is not restricted to embryogenesis but also plays an important role in adults. The discovery of EPCs has led to the concept that angiogenesis—the remodeling or sprouting of preexisting blood vessels—and vasculogenesis may constitute complementary mechanisms for postnatal neo-vascularization.

To what extent and under which conditions bone marrow derived endothelial progenitors contribute to new vessel formation still has to be elucidated. Recent preclinical and clinical

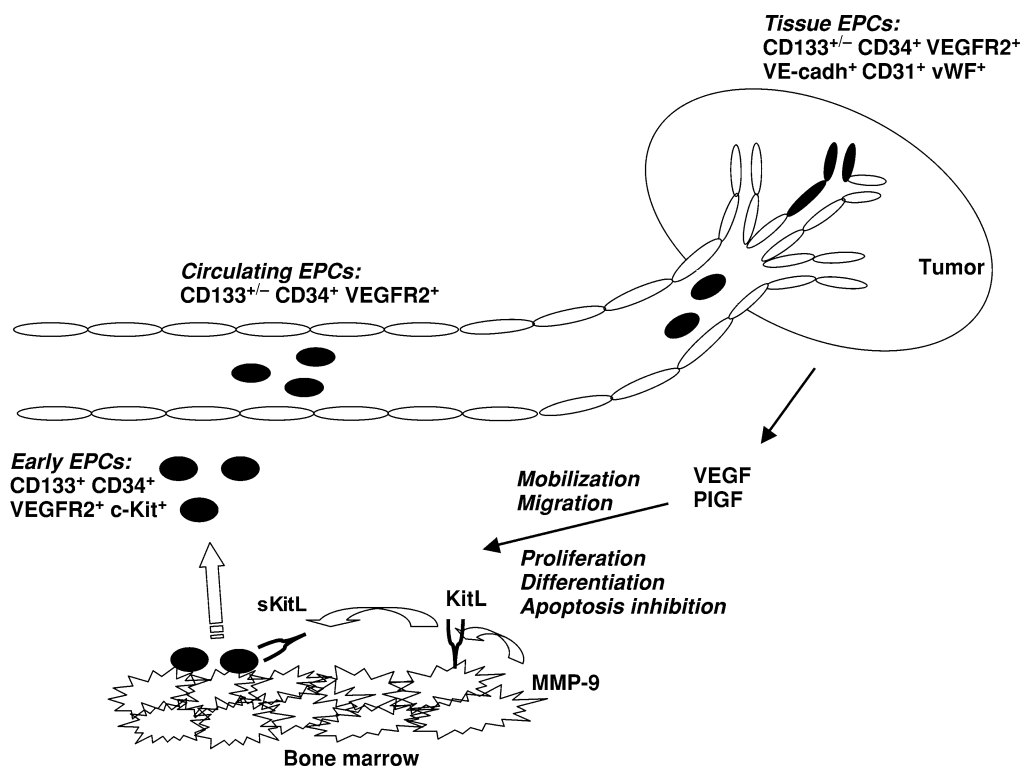


Fig. 1. Markers of EPCs and mechanisms of their recruitment to tumors. EPCs are recruited to tumors by secreted VEGF and PlGF which mobilize EPCs from the bone marrow. These cytokines also have proliferative, antiapoptotic, differentiating, and migratory effects on EPCs. See Subheading 2.2. for details on mobilization mechanisms. EPCs change surface marker expression when they mature from early EPCs to circulating and finally to tissue EPCs.

studies have shown that transplantation of ex vivo expanded EPCs can be successfully used to promote tissue vascularization after ischemic events (13).

Adult EPCs are characterized and isolated by cell surface markers (see Fig. 1). In general, early functional EPCs are characterized by the combined expression of the cell surface markers CD133 and VEGFR2 (16,17). CD133 is an early stem cell marker with as yet poorly understood functions. Whereas VEGFR2 is expressed on both mature ECs and their progenitors, CD133 distinguishes “true” EPCs from mature ECs sloughed from the injured vessel wall and from cells of myelomonocytic origin, which are able to transdifferentiate into endothelial-like cells. Additional markers associated with EPCs include CD34, CD31, VE-cadherin, von Willebrand factor (vWF), CD105, UEA, lectin staining, and uptake of acetylated LDL (13,18). $CD34^{-}/CD14^{+}$ mononuclear cells have the potential to acquire an endothelial phenotype and to coexpress endothelial lineage markers. Infusion of these cells appears to be effective for re-endothelialization after vascular injury (19).

2.2. The Role of EPCs in Tumor Angiogenesis

There is evidence for an important role of bone marrow-derived cells in the neo-vascularization of tumors. Increased numbers of EPCs have been detected in the circulation

of tumor-bearing animals as well as in cancer patients (20). Importantly, transplanted EPCs incorporated functionally into tumor-associated vessels of implanted tumors in different animal models. Studies have taken advantage of transgenic mouse models and transplantation techniques, in which sex-mismatched or genetically marked bone marrow-derived cells were used to distinguish between tumor-induced vasculogenesis and angiogenesis. Asahara et al. transplanted tumor-bearing mice with transgenic bone marrow-derived cells, which were designed to constitutively express a genetic marker under the transcriptional regulation of different endothelial-specific promoters. He demonstrated that neovasculature of the developing tumors comprised marker gene-expressing cells (21). Similar results were obtained in wild type mice transplanted with bone marrow cells expressing green fluorescent protein (GFP) or β -galactosidase or lacking sphingomyelinase (22,23). Lyden and colleagues used Id-mutant mice that did not grow tumors as a result of defective angiogenesis to determine the importance of recruiting EPCs for tumor vasculogenesis. Following transplantation with wild-type bone marrow endothelial and hematopoietic progenitors from the bone marrow significantly contributed to neovascularization and thus restored tumor growth (24). The majority of the newly formed tumor vessels were derived from the genetically marked donor, mainly during the early stages of tumor growth. In contrast to these studies, no contribution of bone marrow-derived cells to tumorigenesis was found by Göthert et al. (25). Another study also did not find an involvement of bone marrow-derived EPCs in tumorigenesis, but defined a subset of bone marrow-derived cells of hematopoietic origin that contributed indirectly to tumor neovascularization (26). Similarly, Rajantie and colleagues failed to detect a contribution of EPCs to tumor vasculogenesis, but provided evidence that bone marrow-derived cells contribute to the formation of periendothelial vascular mural cells (27). These partially conflicting data suggest that whereas bone marrow-derived cells take part in the cellular make-up of tumor vessels, their relative contribution to tumor ECs, periendothelial cells and nonstructural but proangiogenic cells has yet to be determined.

Mobilization and recruitment of EPCs to the tumor vasculature are regulated by a variety of growth factors, cytokines, and surface receptors. Under steady state conditions the number of circulating EPCs in the peripheral blood is low. However, in response to angiogenic stimuli, EPCs are rapidly mobilized from the bone marrow. This process is associated with elevated levels of VEGF in the plasma, suggesting that VEGF is the most critical factor promoting mobilization of EPCs (20,28). VEGF (29,30) and placental growth factor (PIGF), another member of the VEGF family, are released by many tumors. These angiogenic factors induce expression of matrix metalloproteinase (MMP)-9 in the bone marrow microenvironment, which in turn activates the release of soluble Kit ligand (sKitL). Increased levels of sKitL then promote the proliferation and mobilization of stem and progenitor cells into the peripheral circulation (30). Furthermore, VEGF interacts with its receptors, VEGF receptor-1 (VEGFR1, also termed Flt-1), and VEGFR2 (also termed Flk-1 or kinase insert domain receptor, KDR), expressed both on endothelial and hematopoietic stem cells, thereby promoting further maturation of EPCs and the recruitment of these cells to the sites of neovascularization. PIGF—which signals primarily through VEGFR1—also contributes to vessel growth in tumors by recruiting BM derived cells (31). The crucial role of these growth factors in tumor vasculogenesis was further confirmed by studies, in which either

the growth factors or their receptors were inhibited. Inhibition of VEGF production or function by different classes of VEGF antagonists including neutralizing antibodies, soluble receptors, and compounds that block VEGF receptor signaling resulted in the inhibition of tumor growth (29,32). Inhibition of either VEGFR1 or VEGFR2 resulted in vascular malfunction, but could only partially block tumor growth. However, simultaneous inhibition of both receptors led to impaired mobilization of bone marrow-derived cells to the tumor vasculature and was highly effective in retarding tumor growth, indicating that signaling through both receptors is required for tumor vasculogenesis (31,33,34). Emerging evidence suggests that stromal-derived factor 1 (SDF-1/CXCL12) in synergy with VEGF may contribute to the recruitment of EPCs to tumors (33). Little is known about signals that direct incorporation of EPCs into tumor-associated vessels.

2.3. Apoptotic and Antiapoptotic Mechanisms in EPCs

Similar to other cellular vehicles apoptosis of EPCs has to be curtailed during ex vivo expansion and homing to the tumor as well as after activation of its proapoptotic payload. On the other hand, apoptosis of the targeted tumor has to be induced, while normal tissue must be spared.

There is only scant information about the pro- and antiapoptotic mechanisms operating in EPCs. Much relevant information, however, can be gleaned from apoptosis occurring during vessel development and from apoptosis in mature ECs. Angiogenic growth factors not only stimulate EC proliferation and migration, but also concomitantly inhibit apoptosis, thus providing a survival advantage to ECs. VEGF and the VEGF receptor Fkl-1 play a pivotal role in maintaining EC integrity as demonstrated by targeted disruption of these genes (12,36,37). The antiapoptotic effect of VEGF and also of angiopoietin-1 (Ang-1; via activation of its receptor Tie-2) is mainly mediated through activation of the survival promoting PI3K/Akt signaling pathway (38,39). Activation of Akt stimulates the phosphorylation of proapoptotic Bad promoting its sequestration and thus decreasing apoptosis (40). Moreover, Akt activates the endothelial nitric oxide synthase (eNOS). Enhanced synthesis of endothelial NO promotes EC survival by inhibiting the cysteine protease activity of the effector caspases (41). Akt is also known to activate mTOR. mTOR-mediated effects appear to play a role in adult EPCs, because rapamycin-mediated inhibition of mTOR, the target of rapamycin, increases apoptosis and decreases proliferation and differentiation of adult EPCs (42). VEGF has been also shown to mediate the upregulation of antiapoptotic proteins such as survivin, XIAP and Bcl-2 (43–45), the importance of the latter being highlighted by its targeted disruption leading to attenuation of vascular development and of pathological angiogenesis (46). VEGF-activated Akt also mediates down-regulation of p38 mitogen-activated protein kinase (MAPK)-dependent apoptosis (47). Intercellular contacts such as mediated by VE-cadherin (48) also promote EC survival as does CD31 (PECAM-1), which inhibits Bax-dependent mitochondria-mediated apoptosis (49). Cellular receptors interacting with the extracellular matrix can emanate antiapoptotic signals. Thus, $\alpha\upsilon\beta 3$, the receptor for several ligands anchored in the extracellular matrix, triggers antiapoptotic NF- κ B signaling once ligated (50). This activity may form part of the dependence receptor nature of $\alpha\upsilon\beta 3$. The receptor acts antiapoptotic when ligated to ECM-bound ligands by activating the Src, PI3K, and Erk pathways and

by increasing Bcl-2, c-FLIP, and IAP protein activity. The receptor switches to proapoptotic activity involving p53 when triggered by ligands not bound to the ECM (51). This occurs during detachment of therapeutic EPCs from their culture matrix and during homing until the EPCs have integrated into the tumor and are protected against apoptosis again (52). Constitutive overexpression of human telomerase reverse transcriptase (hTERT) rescues adult EPCs from starvation-induced apoptosis, enhances their proliferative capacity, and keeps them in an undifferentiated state (53). Compared with human umbilical vein ECs, adult EPCs derived from blood express significantly higher levels of the antioxidative enzymes MnSOD, catalase, and glutathione peroxidase leading to decreased oxidative stress-induced apoptosis (54). Thus, adult therapeutic EPCs may possess a degree of intrinsic protection against radical producing proapoptotic payloads.

With so many antiapoptotic mechanisms in place, strong stimuli are necessary to induce apoptosis in ECs. Such stimuli comprise, among others, lipopolysaccharides and endostatin (55,56), whose proapoptotic effects are mediated by the mitochondrial pathway. In contrast, death receptor-mediated apoptosis plays a limited role in EC that may be secondary to the high expression of cFLIP (57) and activation of NF- κ B.

From these findings it can be surmised that the default mode of adult ECs is antiapoptotic. This is reflected by the low physiological turnover of ECs in the adult. Marked changes, such as infection, wound healing, hormone withdrawal during the uterine cycle, or tumor growth are required to increase turnover and associated apoptosis of ECs. In addition to these intrinsic antiapoptotic mechanisms, ex vivo expanded EPCs are subjected to antiapoptotic growth factors such as VEGF or bFGF and antiapoptotic extracellular matrices such as collagen and fibronectin. In concert, these mechanisms allow for ex vivo expansion of therapeutic EPCs with minimal spontaneous apoptosis. There are, however, two major proapoptotic insults to EPCs used for tumor therapy. One occurs when EPCs remain in a suspended state following disruption from the matrix of the culture vessel and during homing until they have integrated into the tumor (vessels). As discussed above, the disruption of antiapoptotic integrin signaling renders them susceptible to apoptosis during transit. Because the second major proapoptotic insult in the life of therapeutic EPCs, the activation of its payload, occurs during or shortly after this vulnerable phase, the therapeutic effect of the EPC may be impaired. Increasing protection against apoptosis without diminishing homing and tumor killing efficiency is thus an important goal of further research.

EC apoptosis plays a crucial role in tumor vasculature, because survival of the EC within the tumor vasculature influences the survival of the tumor. Therefore, inhibition of endothelial survival factors or the activation of the EC apoptotic machinery could be an attractive antitumor strategy. This is highlighted by recent work proposing endothelial apoptosis as a key determinant of the tumor response to ionizing radiation treatment. Tumors transplanted into mice deficient in the proapoptotic molecules Bax or acid sphingomyelinase were more resistant to radiotherapy than the same tumors in wild-type animals (23).

2.4. EPCs for Tumor Therapy

The involvement of endothelial progenitors in tumor vasculogenesis provides the rationale to use these cells for therapeutic targeting of the tumor vasculature. Growth and metastasis of the majority of tumors critically depends on the formation of

new blood vessels (58). Because new vessel formation in adults is restricted to wound healing and to the female ovulatory cycle, it has been put forward, that the natural tropism of circulating EPCs could be exploited to deliver and selectively express genes at tumor sites. The use of endothelial progenitors as potential cellular vehicles for tumor gene therapy requires the isolation and *ex vivo* expansion of the cells, followed by their genetic manipulation and subsequent reinfusion into the patients.

2.4.1. Sources of EPCs

EPCs have been derived from mouse embryos, mouse or human embryonic stem (ES) cells, fetal liver, human umbilical cord blood, postnatal bone marrow, and peripheral blood. Embryonic EPCs derived from ES cells have been shown to differentiate into mature ECs and, following transplantation, to contribute to vasculogenesis also in the adult (59). Embryonic EPCs have an unlimited proliferative capacity and are easy to manipulate genetically. In a proof of principle study, the potential of these cells for systemic cancer gene therapy has been explored (60). Given the potential immunogenicity of differentiated ES-cell derivatives and ethical considerations limiting their generation, there is, however, a need for other sources of EPCs.

Umbilical cord blood contains a large number of stem and progenitor cells. In contrast to progenitors generated from adult bone marrow, progenitors derived from cord blood have distinctive proliferative advantages, including the capacity to form a greater number of colonies, a higher cell-cycle rate, and longer telomeres. EPCs generated from human cord blood have been shown to achieve more than 100 population doublings, as opposed to those generated from the mononuclear fraction of adult peripheral blood that stopped dividing after 20 to 30 population doublings (61). Moreover, in contrast with bone marrow, cord blood can be obtained noninvasively. Studies have shown, that transplantation of cord blood-derived EPCs efficiently augments postnatal neovascularization *in vivo* (62).

Adult endothelial progenitors have been initially isolated from CD34-positive peripheral blood mononuclear cells (13). Since their identification a number of studies have reported more defined methods of isolation and culture. However, because most of the studies have focused on promoting the differentiation of isolated EPCs into mature, terminally differentiated ECs, conditions required for the long-term proliferation, and maintenance of undifferentiated EPCs still have to be elucidated. Recently, Reyes and colleagues identified a unique cell population from postnatal bone marrow that has extensive proliferation potential and the capacity to differentiate into different cell types of the mesodermal lineage (63). These nonendothelial stem cells, termed multipotent adult progenitor cells (MAPCs), were able to differentiate into ECs in the presence of VEGF *in vitro* and to contribute to vasculogenesis *in vivo*. Remarkably, intravenous injection of undifferentiated MAPCs resulted in new vessel formation in transplanted tumors, indicating that MAPCs can differentiate into ECs *in vivo*, in response to local clues. The authors proposed MAPCs as the precursors of EPCs and as a promising source of ECs for therapeutic approaches.

2.4.2. *In Vitro* Expansion of EPCs

EPCs have been isolated from mononuclear cells by antibodies against surface markers like CD133 or VEGFR2—coupled to magnetic microbeads—or by adherence culture of unfractionated peripheral blood mononuclear cells (PBMCs). Isolated EPCs are grown

on fibronectin or collagen-coated dishes in the presence of growth factors, such as VEGF, basic fibroblast growth factor (bFGF), and insulin-like growth factor (IGF) and can be differentiated into mature adherent ECs (13,18). Upon endothelial differentiation EPCs downregulate CD133 expression and start to express mature endothelial-specific markers, such as VEGFR2, VE-cadherin, platelet EC adhesion molecule (PECAM-1, CD31), von Willebrand factor (vWF), and CD105.

The success of therapeutic approaches depends on the isolation of a sufficient number of EPCs for expansion. As described previously, the number of EPCs in the bone marrow and in the circulation is low. However, clinical studies have demonstrated that the number of circulating EPCs can be significantly increased by injecting chemotactic factors, such as VEGF, PlGF, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (64). Drugs such as statins can also be used to mobilize larger number of EPCs for in vitro manipulation (65). Lin and colleagues described a reliable method for isolating a virtually unlimited number of bone marrow-derived EPCs (66). After long-term culture of PBMCs late outgrowth ECs with high proliferative capacity were generated. In contrast, vessel wall-derived, or circulating mature ECs gave rise to early outgrowth ECs with limited proliferation potential, thereby providing a functional assay to discriminate bone marrow-derived EPCs from contaminating ECs. Late outgrowth cells, termed as blood outgrowth endothelial cells (BOECs) could be passaged up to 30 times.

2.4.3. Genetic Manipulation of EPCs

Ex vivo expanded EPCs derived from different sources are easily amenable to genetic manipulation. Transduction of EPCs and their putative precursors with retrovirus (22,67) and lentivirus (26) vectors have been shown to be feasible and resulted in long-term transgene expression in mice. These vectors mediate stable genomic integration of the transgene, thus allowing for long-term transgene amplification. This may be necessary for several therapeutic approaches using EPCs as cellular vehicles. Moreover, replication-defective retro- and lentiviral vectors do not express any viral proteins, thereby decreasing the chance of immune clearance of the transduced EPC following readministration to the host.

EPCs or other bone marrow-derived cells may also be manipulated using nonintegrating vectors, such as those based on adenovirus and herpes simplex virus (HSV). Such vectors are advantageous if an antitumor effect can be achieved within a short interval.

2.4.4. EPCs as Cellular Vehicles for Tumor Gene Therapy

Recent advances in the biology of EPCs have suggested the feasibility of their use as cellular vehicles. As described previously, EPCs can be easily isolated and ex vivo expanded in quantities required for therapeutic approaches. Cells can be efficiently transduced with therapeutic payloads using viral vectors. What renders EPCs attractive cellular vehicles for systemic tumor gene therapy is their capacity to localize not only into the tumor proper but also into the tumor's vasculature.

Tumor vessels are promising targets for anticancer therapeutics for several reasons. The strict dependence of the majority of tumors on recruiting new blood vessels to grow and metastasize provides a common target for the treatment of different tumor types irrespective of their antigenic and genetic make-up. Furthermore, in adults—with the exception of wound healing and the uterine cycle—neovascularization is restricted to

tumor growth. In addition, tumor vessels are accessible to circulating EPCs. This might eliminate some of the major obstacles associated with targeting of solid tumors, such as poor tissue penetration. It has to be noted, however, that systemically administered EPCs incorporate into only a minority of tumor vessels (21,22,63,67), which limits the ability of therapeutic EPCs to destroy tumor vessels. However, because EPCs have been shown to home also to the tumor proper, a second venue to attack the tumor is open. Therefore, as far as the ability to target tumors as a whole is concerned, the relative contribution of EPCs to the formation of tumor vessels is a somewhat moot point.

2.4.5. *EPCs Can be Fitted With Apoptosis Effectors*

2.4.5.1. SUICIDE GENE BEARING EPCs

Antitumor suicide gene therapy is one of the emerging strategies against cancer. Suicide genes, which encode nonmammalian enzymes, are capable of converting non-toxic prodrugs into highly toxic metabolites. The successful eradication of the tumor depends on the ability of the suicide gene-expressing target cells to exert cytotoxicity on neighbouring tumor cells, thereby providing a potent bystander effect (68). Several groups have investigated the feasibility of using suicide gene-expressing bone marrow cells and EPCs as gene-targeting vectors to mediate systemic antitumor responses.

CD34⁺ cells transduced by a TK-encoding HSV vector have been shown to migrate to a skin autograft in a nonhuman primate model and to mediate accelerated regression of the graft following ganciclovir (GCV) treatment (69). Genetically modified CD34-positive cells have also been tested by the same group for the ability to exert a strong cytotoxic bystander effect (70). Transduced cells expressed the TK gene with high efficiency, became sensitive to the effect of GCV, and mediated a strong bystander killing of both tumor cells and proliferating ECs in vitro.

Bone marrow-derived endothelial progenitor-like cells transplanted into tumor-bearing mice incorporated into the angiogenic vasculature of the growing tumors (67). EPCs contributed to between 10 and 25% of the tumor-associated vasculature in sublethally irradiated mice, whereas the number of donor EPCs was far smaller in the tumor vessels of nonirradiated mice. This observation underlined the hypothesis that systemically administered EPCs might have to compete with endogenous EPCs, thus suppression of endogenous EPCs by irradiation may be necessary for optimal donor EPC incorporation. When the EPCs were modified to express the TK gene, they induced a strong antitumor effect without any systemic toxicity after GCV treatment, pointing to the feasibility of employing EPCs as angiogenesis-selective cancer-targeting vectors.

DePalma and co-workers transplanted bone-marrow progenitors expressing the TK gene under the control of an endothelial-specific promoter/enhancer (Tie2) (26). As described above, these cells homed specifically to tumors and interacted with vascular ECs. The EPCs and tumor bystander cells were selectively eliminated following GCV treatment, resulting in slower tumor growth without systemic toxicity.

More recently it has been shown that murine embryonic EPCs administered systemically preferentially home to hypoxic areas of lung metastases (60). Because embryonic EPCs do not express MHC I proteins and are not killed by nonactivated NK cells, they are able to evade immunological rejection. When modified to express the cytosine deaminase gene, these embryonic EPCs exerted a bystander effect on tumor cells upon administration of 5-FC, thus prolonging the life of tumor bearing mice.

2.4.5.2. ANTIANGIOGENIC EPCs

An increasing number of clinical trials (phase I, II, and III) have demonstrated the efficacy of antiangiogenic compounds for the treatment of cancer (71). Gene-mediated delivery of these agents has been proposed to have potential benefits such as generation of high-drug concentrations limited to the tumor. This notion was supported by recent clinical data showing that transplantation of unfractionated bone marrow cells engineered to express an angiogenesis inhibitor—a truncated form of VEGFR2—resulted in long-term expression of the therapeutic protein in the tumor in mice and in reduced tumor growth (22).

2.4.5.3. EPCs AS PACKAGING CELLS FOR VIRAL VECTORS

EPCs, as other cellular vehicles, can be engineered to produce viral vectors (72,73). This elegant method avoids systemic exposure to viral vectors and protects them from immune inactivation, nonspecific adhesion, and other causes of particle loss during transit to the tumor. Using such vector-producing cells appears to be an attractive strategy if the incorporation efficiency of the particular cell carrier is low. Each cell can release multiple virus particles at the tumor site, thereby amplifying the therapeutic effect.

2.4.6. Clinical Translation

Because of the availability and ease of isolation, expansion and manipulation, and because of immunological tolerance, EPCs derived from autologous blood will most likely be the first choice as the source of therapeutic EPCs. However, several points have to be addressed before autologous EPC-based tumor gene therapies can enter clinical trials. First, to yield an adequate therapeutic effect a sufficient number of these cellular carriers has to reach the tumor site. As discussed above, the number of endogenous EPCs recruited from the bone marrow into tumors appears to be low, as is the number of these EPCs actually incorporating into tumor vessels. The same holds true for ex vivo expanded autologous EPCs in preclinical tumor models. Thus, data on the tumor homing efficacy of systemically injected autologous EPCs in patients are required but are not yet available. Furthermore, a better understanding is needed as to what degree of differentiation is required for ex vivo expanded autologous therapeutic EPCs to achieve optimal tumor homing and whether genetic manipulation of the EPCs is necessary to increase homing. In addition, it has to be investigated whether myelosuppression to suppress recruitment of endogenous EPCs that potentially compete with therapeutic EPCs may prove beneficial.

Second, recruitment of EPCs depends on the tumor microenvironment, which may vary not only between tumor entities but also within a given tumor. An obvious intratumoral variable is the degree of hypoxia that leads to differential recruitment of EPCs predominantly into hypoxic areas (60). More investigations are required to define tumor entities or tumor states that are dependent on the recruitment of EPCs and to delineate the molecular mechanisms governing EPC recruitment to tumors.

Third, although it appears that EPCs are partially protected against apoptosis, further research is needed to elucidate how to protect them better against their proapoptotic payload without sacrificing the reliability of the elimination of EPCs at the end of therapy. Final elimination is needed to avoid an ongoing proangiogenic effect of surviving EPCs on the tumor and to preclude the remote possibility of malignant transformation of the highly proliferative EPCs.

The reward of these efforts may be a cellular vehicle that provides efficient, safe, and specific systemic delivery of highly proapoptotic effectors into tumors.

3. Neural Progenitors for Brain Tumor Gene Therapy

3.1. Gene Therapy of Brain Tumors

Brain tumors are hard to treat. In particular the infiltrative nature of many of these tumors, such as glioblastoma multiforme, often makes it impossible to achieve complete surgical resection or to apply curative radiotherapy without risking severe side effects. Brain tumors are also often chemoresistant. Thus, novel therapies are urgently needed for these tumors.

Brain tumors often have characteristic genetic alterations in the form of activated oncogenes or disrupted tumor suppressor genes. Gene therapy holds the promise of augmenting conventional brain tumor therapy by specifically targeting these genetic alterations. However, such approaches require a degree of gene transfer and targeting efficiency impossible to achieve with current vector systems. Therefore strategies have been developed which are independent of the genes specifically altered in brain tumors allowing more leeway in gene transfer efficiency and targeting. The effectors of these strategies include suicide/prodrug systems, toxins, cytokines, and angiogenesis inhibitors. But even such spatially broad-acting effectors require a degree of targeting efficiency hard to obtain. It is thus not surprising that cellular vehicles with brain tumor homing abilities, such as neural stem or progenitor cells, have recently received increasing attention.

3.2. The Physiological Role of Neural Progenitor Cells

During the embryonic and early postnatal period, a large number of cells in the central nervous system (CNS) are born. These neurons are derived from self-renewing multipotent neural stem and progenitor cells that are highly migratory. For a long time the adult brain had been considered to be postmitotic. Recently, however, neural progenitor cells (NPCs) have been shown also to exist in the adult CNS (74,75). In the adult brain of rodents these NPCs are found within the subventricular zone (76), the dentate gyrus of the hippocampus (77), the septum and striatum (78), the spinal cord (79), the neocortex and the optic nerve (80). Adult neurogenesis has also been found in nonhuman primates (81) and in humans (82).

3.3. NPCs for Brain Tumor Therapy

3.3.1. NPCs Home to Brain Tumors

NPCs from various sources and from various species are highly migratory when responding to environmental cues during development, following injury, or in the presence of brain tumors. NPCs from nonhuman primates can migrate over a distance of more than 2 cm (83). Similarly, human fetal neural stem cells migrate extensively when injected into the brain of immunodeficient neonatal mice (84). Human fetal NPCs have also been shown to migrate towards ischemic areas in the rat cerebral cortex with migrating NPCs expressing the neuroblast marker doublecortin, whereas the majority of NPCs that have stopped migration at the lesion border express the early neuronal marker beta-3-tubulin (85).

What has kindled the interest of oncologists is the marked homing efficiency of NPCs to tumors. Aboody et al. showed that human and murine NPCs home specifically to gliomas implanted in mice even when the NPCs are injected into the hemisphere contralateral to the tumor (86). In addition, NPCs appeared to “chase down” individual tumor cells “escaping” from the main tumor mass into normal brain tissue. A similar tropism of NPCs towards both the main tumor mass and tumor satellites of experimental glioblastomas was demonstrated by Ehteshami et al. (87). Both glial-restricted progenitors and ES cell-derived neural stem cells have been shown to migrate towards experimental gliomas (88) as have been neural stem-like cells expanded from human adult bone marrow (89). Furthermore, NPCs homed to brain tumor even when they were administered outside the brain by intravenous injection (90). The migratory tropism of NPCs to experimental brain tumors has been monitored noninvasively in vivo using luciferase-labeled NPCs and bioluminescence imaging (91). Systemically administered bone marrow cells labeled with superparamagnetic iron oxide nanoparticles could be detected in the tumor vessels of mouse glioma by in vivo magnetic resonance imaging (92). Such techniques allow NPC-based therapies to be monitored for safety and efficacy.

The mechanisms underlying the migration and homing of NPCs have only begun to be elucidated. Thus, CXCR4-bearing NPCs have been shown to migrate to SDF-1 α /CXCL12 expressing ischemic brain areas (93). Information about other interactions may be gleaned from the migration of glioma cells. Because these cells express similar receptors as NPCs they may be targets for the same migration-inducing chemoattractants.

3.3.2. NPCs Can be Isolated From a Variety of Sources and Expanded Ex Vivo

NPCs can be obtained or derived from two sources: either from nonregional cells such as embryonic stem cells and bone marrow cells or from regional tissue stem cells. NPCs can be isolated and propagated from many neural tissues at any stage after the formation of neuroectoderm (i.e., from embryonic, fetal, or adult CNS). After isolation, NPCs are grown in serum-free medium as clonally derived proliferating floating aggregates, so-called neurospheres. NPCs can be expanded using mitogens such as fibroblast growth factor 2 (FGF-2) and epidermal growth factor (EGF) and cytokines including leukemia inhibitory factor. NPCs may also be expanded through introduction of an immortalizing gene. Expanded NPCs can be differentiated into neurons, astrocytes and oligodendrocytes (77) which are capable of integrating into neural structures (84,94–97). This raises the vision of NPCs being used not only as cellular vehicles for brain tumor therapy, but also to mitigate neural loss caused by the tumor or its therapy.

Adult NPCs have been derived from the brain of adult humans (98). Availability of autologous NPCs from the adult human brain is limited because of the invasive nature of their procurement and the dearth of dispensable brain tissue. Supply of brain tissues from cadavers and fetuses is also limited and carries the risk of immunological rejection.

NPCs can be generated from ES cells of various species, including rodents (99,100) nonhuman primates (101,102), and humans (103). Although this source of NPCs is potentially unlimited, immunogenicity, the potential for malignant transformation, and ethical considerations may limit their use as cellular vehicles.

Immortalized NPC cell lines have been generated from mouse (104), rat (105), and human (106–108). Although immortalized NPC lines are useful for experimental purposes, the potential malignant transformation precludes their clinical use.

Progenitor cells that are more easily accessible, such as hematopoietic stem cells from the bone marrow, may substitute progenitor cells that are difficult to access for autologous grafting, such as brain-derived NPCs. Several groups have demonstrated by immunohistochemistry that bone marrow cells can be differentiated into neurons and glial cells in vitro and in vivo (109–111). Although this concept of “transdifferentiation” has been challenged (112), such cells may still be useful as autologous cellular vehicles for brain tumor gene therapy, because experimental brain tumors were successfully treated using neural stem-like cells expanded from human adult bone marrow and transduced with platelet factor 4 (PF4), an antiangiogenic protein (89).

For brain tumor gene therapy employing NPCs it will be important to determine not only from which source NPC should be derived from, but also which stage of differentiation is to be used. For example, neural stem cells derived from neurospheres from the subventricular zone may have a higher capacity for expansion but a decreased ability to migrate compared to more differentiated NPCs from the same area (113).

3.3.3. Apoptotic and Antiapoptotic Mechanisms in NPCs

Apoptotic mechanisms are operational in gene therapy using neural progenitors in several aspects. First, spontaneous apoptosis occurring in NPCs has to be controlled if a sufficient number of NPCs is to be efficiently generated in vitro and delivered in vivo. Second, NPCs acting as cellular vehicles have to be protected from apoptosis while homing to the tumor. Third, NPCs should be resistant to apoptosis induced by their proapoptotic payload. Fourth, apoptosis is a crucial mechanism of cell death of the cancer cells targeted by therapeutic NPCs. Finally, normal brain tissue should be resistant to apoptotic insults induced by NPCs.

Despite the obvious importance of apoptosis in NPC gene therapy little is known about it. Indirect clues can be gained by extrapolating from developmental apoptosis during brain development. During this process an excess of neural progenitors is born requiring widespread apoptosis to form the definitive neural structures. The molecular mechanisms underlying this process have just begun to be elucidated. Developmental apoptosis in embryonic NPCs depends on Apaf1, caspase-9, and caspase-3, but is independent of Bax and Bcl-X_l (114–118). Notch signaling reduces the embryonic NPC pool by inducing p53-dependent apoptosis in embryonic NPCs (119). Notch signaling also induces embryonic NPCs to differentiate (120,121). Embryonic NPCs are protected against death receptor mediated apoptosis since caspase-8 is absent and because the death receptor inhibitory protein PED/PEA-15 is highly expressed in embryonic NPCs (122). An interesting mechanism of controlling the number of embryonic NPCs is the asymmetric distribution of proapoptotic molecules such as prostate apoptosis response (PAR)-4 and endogenous ceramide into only one of the daughter cells during mitosis (123). Using a ceramide analog, this mechanism can be exploited to induce selective apoptosis in nondifferentiated pluripotent stem cells, thus enriching ES cell-derived NPCs (124). Taken together, these data show that apoptosis of embryonic NPCs proceeds via the intrinsic apoptosis pathway with caspase-3 being the pivotal executioner. Spontaneous apoptosis of therapeutic embryonic NPCs may follow the same

pathway. In contrast, apoptosis of embryonic NPCs induced by DNA-damaging agents and γ -irradiation depends on p53 and caspase-9, but neither on Bax nor caspase-3 (125). Rendering embryonic NPC deficient in p53 and caspase-9 may thus induce protection of therapeutic embryonic NPC against apoptosis-inducing DNA-damaging payloads.

In adult NPCs Bax and Bak increase apoptosis and differentiation of NPCs without decreasing their production or proliferation (126,127). Protection against receptor-mediated apoptosis is provided by the absence of caspase-8 and overexpression of PED/PEA-15 (122). Thus, similar to embryonic NPCs, the intrinsic apoptosis pathway appears to be pivotal in programmed cell death of adult NPCs. Unlike in embryonic NPCs, however, Bax and Bak play important roles. Inhibiting the intrinsic or the common execution pathway of apoptosis should protect NPCs from apoptosis and thus increase their therapeutic value. Haploinsufficiency for PTEN in adult NPCs increases phosphorylation of AKT, rendering NPCs more resistant to oxidative stress-induced apoptosis (128). Interestingly, adult NPCs are exquisitely sensitive to γ -irradiation-induced apoptosis (129), which is associated with ROS production and may involve p53 (130). Similar apoptosis regulating mechanisms may be operational in spontaneous apoptosis of adult therapeutic NPCs.

3.3.4. NPCs are Partially Protected From Immune Responses

Several factors contribute to a relative immune privilege of NPCs within the brain. The brain *per se* is to some extent an immune-privileged site. Although leaky in pathological states such as malignancies, the blood–brain barrier (BBB) does donate some degree of immune protection by virtue of limiting transgression of lymphocytes. The brain also has few lymphatics and brain tumors often secrete immunosuppressive proteins such as tumor growth factor (TGF)- β . In addition, NPCs themselves have features causing immunoprotection. EGF-responsive NPCs from postnatal day 1 mice do not express major histocompatibility complex (MHC) I and II antigens and survive for a prolonged period of time at a nonimmune-privileged site in an allogeneic recipient (131). Although differentiation was not reported to increase MHC I and II, an increase was reported after exposure to γ -interferon. Taken together, these data raise the possibility that NPCs can be employed even in an allogeneic setting, which would markedly increase the utility of ES cell-derived NPCs.

3.3.5. NPCs Can be Genetically Modified With Apoptosis Effectors

3.3.5.1. SUICIDE GENE BEARING NPCs

One promising brain tumor therapy is augmentation of the specific cytotoxicity of anti-cancer drugs by selectively increasing their concentration within the tumor through on-site conversion from nontoxic prodrugs. This gene-directed enzyme/prodrug system is also called suicide gene therapy. Given the low gene transfer efficiency *in vivo* this strategy would be futile if all cells of a tumor had to be transfected. What makes suicide gene therapy an option is the so-called “bystander effect,” that is, the killing of untransfected cells surrounding the transfected target cells. Many suicide gene/prodrug systems have been investigated in preclinical trials. One of the first and most widely used systems is the herpes simplex virus thymidine kinase (TK) gene with the prodrug GCV. HSV-TK phosphorylates nontoxic GCV to intermediates that are metabolized to a toxic form, which is incorporated into replicating DNA causing apoptotic cell death (132). TK/GCV-

mediated apoptosis is characterized by CD95-ligand-independent CD95 aggregation leading to the formation of a Fas-associated death domain protein (FADD) and caspase-8-containing, death-inducing signaling complex (DISC) (133). Mitochondria amplify TK/GCV-induced apoptosis by regulating p53 accumulation and the effector phase of apoptosis (134). TK/GCV-induced apoptosis of tumor cells is augmented by TNF-related apoptosis-inducing ligand (TRAIL) depending both on caspase activation and on mitochondrial apoptogenic function (135). Although gene therapy of glioma using TK/GCV has, with few exceptions (5,6,136), failed in the clinic as a result of low transduction efficacy (2,3,137), this problem may be circumvented by using the highly migratory NPCs expressing HSV-TK, as suggested by a preliminary *in vitro* study (138). TK/GCV, however, depends on the presence of intercellular gap junctions, which may limit the killing effect of NPCs to tumor cells in the immediate vicinity.

Another commonly used suicide gene prodrug system is cytosine deaminase/5-fluorocytosine (CD/5-FC). CD deaminates the nontoxic pyrimidine 5-fluorocytosine to the cytotoxic 5-fluorouracil (139). Aboody and colleagues were the first to combine an enzyme/prodrug system and NPCs for brain tumor gene therapy. Murine NPCs expressing CD migrated to tumors and microscopic satellites and significantly reduced the tumor mass upon administration of the prodrug (86). Immortalized CD-expressing NPCs coinjected with glioma cells into the brain of rodents significantly decreased tumor size after application of 5-FC (140). 5-FU diffuses out of the cell harboring CD and into neighbouring tumor cells independent of gap junctions, thus making this suicide gene system more versatile and probably also more efficient than TK/GCV.

3.3.5.2. CYTOKINE SECRETING NPCs

Malignant gliomas are able to overcome host immune defences through a variety of mechanisms. Most clinical immunotherapy protocols have manipulated the immune response in an attempt to direct it against cancer cells. One approach is stimulation with cytokines to enhance recognition and thus eradication of the tumor cells. Interleukin (IL)-2, IL-4, IL-12, and GM-CSF have been used for this purpose in animal models.

NPCs that migrate to the tumor and secrete tumoractive cytokines constitute an attractive therapy modality. Mouse primary neural progenitor cells transduced with the IL-4 gene and injected into established syngeneic brain glioblastomas elicited a tumor-directed T-cell response with a strong antitumor effect (141). This effect was more pronounced than the effect of retrovirus-mediated *in vivo* transfer of IL-4. Similarly, NPCs derived from fetal forebrain infected with an adenoviral vector carrying the gene for IL-12 prolonged survival of intracranial GL26 glioma-bearing mice (142). This was associated with enhanced T-cell infiltration into tumor microsatellites and long-term antitumor immunity.

3.3.5.3. NPCs SHEDDING ONCOLYTIC VIRUSES

An increasing number of oncolytic virus vectors have been developed lately for cancer therapy. HSV, one of the first oncolytic viruses to be used for this purpose, can be genetically engineered to replicate and spread selectively in tumor cells while sparing normal neurons (143). Other oncolytic viruses, such as adenovirus and measles virus, have also been shown to be effective in lysing brain tumor cells. However, delivery of viral vectors to tumors in the brain remains a challenge, especially systemic delivery. To meet this challenge, NPCs have been employed as packaging cells. These deliver

HSV-1 mutants that only replicate in dividing (i.e., tumor) cells into established glioma in the mouse (144). Replication of the mutant HSV-1 was blocked in the NPCs prior to implantation into the tumor by chemical growth arrest. NPCs were observed to migrate extensively throughout the tumor and into the surrounding parenchyma, with appropriate gene expression of HSV-1. Thus, NPC-mediated viral vector delivery has the potential to increase the reach of viral vectors within the brain and to magnify the efficacy of viral-mediated gene transfer into diffusely growing brain tumor cells.

3.3.5.4. NPCs EXPRESSING TRAIL

TRAIL is a member of the tumor necrosis factor protein superfamily. It selectively induces apoptosis in a variety of neoplastic cells without evident toxicity to normal cells.

Murine fetal brain NPCs adenovirally infected to release TRAIL migrate to glioblastoma xenografted into the brain of mice, track down their satellites and, by inducing significant apoptosis of both the main tumor and its satellites, cause a marked inhibition of tumor growth while sparing normal brain tissue (87). Importantly, the NPCs are not killed by the TRAIL they produce, highlighting the preferential killing of malignant cells by TRAIL. Using TRAIL as an apoptosis effector thus has the important advantage of leaving the cellular vehicle unharmed. Similar, NPCs differentiated from adult mouse bone marrow and transduced with TRAIL have been shown to migrate to glioblastoma xenografts and induce apoptosis in the tumors, thus prolonging the life of the tumor-bearing mice (89).

3.3.5.5. ANTIANGIOGENIC NPCs

Brain tumors, like other cancers, require angiogenesis to grow. Angiogenesis can be inhibited via diffusible factors. NPCs that home into brain tumors are good candidates for delivering such antiangiogenic factors within or surrounding the tumor mass and its satellites. Human adult bone marrow-derived neural stem-like cells modified to secrete platelet factor 4 (PF4) homed to glioblastoma xenografts, reduced their microvascular density, and prolonged the life of the mice (89).

3.3.5.6. INTRINSIC ANTITUMOR EFFECTS OF NPCs

Interestingly and for unknown reasons even NPCs that have not been engineered to express cytotoxic or immunomodulatory effectors have a growth inhibiting effect on rodent glioma leading to prolongation of the life of glioma bearing rodents (145).

3.3.6. Clinical Translation

Whereas proof of principle has been achieved using NPCs as cellular vehicles for brain tumor gene therapy, clinical relevance still has to be proven. Human brain tumors differ in several important aspects from the rodent mouse tumor models employed in the proof of principle studies. First, the human brain is many times larger than the rodent brain. This translates into much greater distances the therapeutic NPC must cover. Second, the large size of human tumors also challenges both the expandability and the infiltrative ability of NPCs. Third, human brain tumors are often infiltrative, a feature often not recapitulated in rodent brain tumor models. Fourth, in experimental tumors, especially in those of rodent origin, the proportion of cycling cells is higher than in

patient tumors. Cycling tumor cells are usually easier to treat than quiescent ones. Fifth, most experimental tumors are naive and thus more susceptible to therapy, whereas many patients who might be considered for NPC-based therapy will be heavily pre-treated. Finally, neural stem cells, and to a lesser degree progenitor cells, share certain characteristics with tumor cells such as self-renewal, migration, and maybe apoptosis resistance. Thus, these cells may be prone to malignant transformation (146) and it has to be assured that therapeutic NPCs are reliably killed after they have fulfilled their therapeutic function. These caveats notwithstanding, NPCs do constitute a very promising means to deliver therapeutic genes including proapoptotic ones to infiltrative growing brain tumors.

Acknowledgments

This work was supported by grants of the DFG and the Wilhelm Lander-Liftung (to CB).

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Apoptosis and Cancer Therapy

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Summary

The observation that tumor development frequently is accompanied by apoptotic defects and the meanwhile accepted view that the vast majority of conventional anticancer agents primarily cause DNA damage to subsequently initiate the apoptotic machinery, have led to the conclusions that the capability to undergo apoptosis must contribute to the outcome of cancer therapy and that mutations compromising apoptosis might confer chemoresistance. However, experimental results from cell-culture-based systems and conflicting data from the clinic have prompted reasonable doubts about a critical role of apoptosis in cancer therapy. This chapter highlights the technical difficulties in properly assessing the impact of treatment-induced apoptosis, discusses the value of more complex model systems, and underscores the relevance of apoptosis by providing insights from mouse models harboring tumors with genetically defined lesions and clinical observations based on novel proapoptotic compounds.

Key Words: Apoptosis; chemotherapy; mouse models; resistance; senescence; targeted therapies.

1. Introduction

About one hundred years ago, Paul Ehrlich expressed his dream of “magic bullets”—therapeutics that would selectively eliminate harmful microbes or tumor cells (*1*). In the 1940s, the first chemotherapeutic compound active against lymphomas, nitrogen mustard, was discovered as a byproduct of studies interested in elucidating the biological effects of mustard gas, a chemical warfare agent used during World War I (*2*). During the following decades, empirical screens rather than rational efforts of drug development, added novel compounds to the growing arsenal of anticancer agents. Given their inherent toxicity to non-neoplastic cells, these drugs are conceptually far away from Ehrlich’s “magic bullet” vision that anticipated the era of truly targeted therapies. Despite their different pharmacological properties and interference with a variety of molecular targets, most of the conventional anticancer compounds exert their cytotoxic action by producing direct or indirect damage of genomic DNA. Ironically, neither the role of DNA as the prime target of chemotherapeutic drug action, nor the discovery that genes are composed of DNA, nor the causal role of mutant DNA in oncogenic activation or tumor suppressor inactivation was appreciated in the early days of chemotherapy (*3*). Even when DNA was recognized as the critical drug-damaged structure, it was believed for long periods of time that the extent of DNA damage delivered to fast growing cancer cells must be invariably lethal *per se*. Accordingly, the clinical problem of

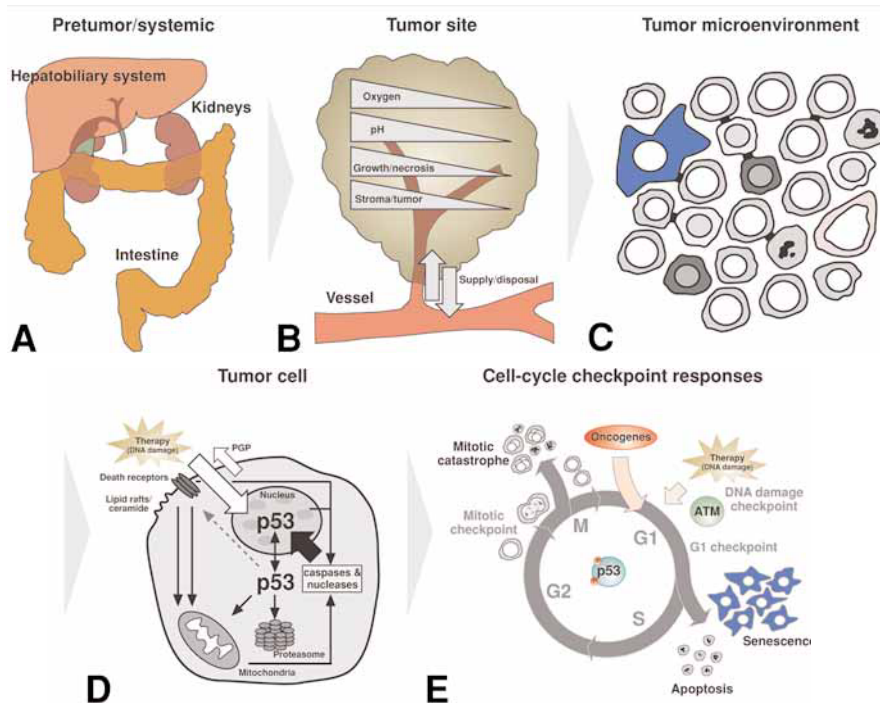


Fig. 1. The road to drug-induced cellular effector programs. **(A)** Upon systemic application, DNA damaging anticancer agents undergo pharmacological modification, for example hepatic toxification, and metabolization with subsequent excretion via the hepatobiliary system, the intestine and the kidneys. **(B)** Depending on the vascularization of the tumor, a fraction of the active drug is delivered to the tumor site, where it will reach individual parts of the tumor to varying degrees. In addition, gradients in oxygen supply and pH, as well as inhomogeneities between tumor and stromal cells and also between proliferating and necrotic areas will influence local drug activity. **(C)** The tumor microenvironment modulates drug action in many ways. Besides the local density of capillary vessels important for drug supply and metabolite disposal, nonneoplastic bystander cells secrete growth factor-like cytokines or directly interact via ligand-receptor mechanisms with tumor cells and alter their apoptotic threshold. Moreover, apoptotic bodies and senescent tumor cells not yet cleared may also impact on the susceptibility of tumor cells to the cytotoxic effects of anticancer agents. **(D)** If they are not instantly eliminated by cellular efflux pumps such as the P-glycoprotein (PGP) most anticancer agents, either in a direct or indirect manner, cause DNA damage. The prime consequence of DNA damage is the initiation of a p53-mediated mitochondrial apoptotic cascade ultimately activating caspases and nucleases. Primarily via changes in the cell membrane, via binding of specific ligands, or secondarily via crosstalk from the intrinsic apoptotic cascade, the extrinsic death-receptor-mediated pathway may augment proapoptotic signaling. **(E)** Cell-cycle checkpoints, sensitized by activated oncogenes, will translate the ATM-sensed DNA damage into p53-mediated pro-apoptotic signals, if the apoptotic machinery is available. As an alternative response, DNA damage may provoke in apoptotically incompetent cells a terminal cell-cycle arrest, termed cellular senescence. Some cells might continue to cycle for another few nuclear divisions, before entering mitotic catastrophe, an attenuated form of apoptosis often executed out of mitosis.

chemoresistance was inferred to mechanisms that may blunt drug action prior to the occurrence of DNA damage, such as accelerated systemic drug metabolism or induced expression of membrane-inserted drug efflux pumps (4,5).

Following up on the discovery of apoptosis as a form of genetically encoded, programmed cell death (6), and the observation that DNA damage may act as an inducer of apoptosis (7), it was subsequently demonstrated that biochemically unrelated classes of DNA damaging anticancer agents may initiate apoptosis as a unique downstream effector cascade (8). In turn, genetic alterations such as overexpression of the strictly anti-apoptotic molecule Bcl2 were shown to produce multidrug resistance to a variety of anticancer compounds in short-term assays *in vitro* (9). Hence, programmed cell death was inaugurated as a novel concept of anticancer drug action based on a common post-damage drug effector mechanism, and genetic defects in this program became candidates for causing chemoresistance. However, despite the attractiveness of this model, the actual contribution of apoptosis to the outcome of cancer therapy still remains unclear.

2. Apoptosis and the Outcome of Therapy

Meanwhile, there is an enormous body of scientific work related to the field of “apoptosis and therapy.” Unfortunately, imprecise use of either term makes it particularly difficult to judge the actual impact of apoptosis on the outcome of therapy.

Apoptosis is a complex sequential process of genetically determined self-destruction that ultimately leads to the activation of proteases with certain substrate specificities, the caspases, and nucleases that produce membrane blebs, degrade DNA into nucleosome-sized fragments and condensate cellular compartments (10). The remainders, so called apoptotic bodies, are subject to phagocytosis devoid of a harmful inflammatory response. Paralleling the increasing molecular understanding of the processes involved in programmed cell death, numerous aberrant forms of apoptosis, termed atypical, caspase-independent apoptosis (11), attenuated programmed death as a consequence of mitotic catastrophe (12), autophagy (13,14), or the possibility of programmed necrosis (15) have been described during the past few years. As a consequence, proper assessment of apoptotic features by experimental procedures has become an issue of debate, and many reports claiming to study apoptotic cell death simply measure viability as a surrogate marker.

Chemotherapeutic agents applied to a cancer cell in a Petri dish and to a cancer patient may not necessarily produce comparable biological effects. Surprisingly, many studies based on the short-term exposure of cancer cells to a cytotoxic compound in culture mutually assume that their findings establish a biologically meaningful approximation of a patient’s outcome to therapy. In fact, only a very limited number of studies addressed the critical question to what extent the apoptotic sensitivity of primary tumor cells measured in a short-term *in vitro* assay may accurately predict the long-term response of the corresponding cancer patient. Remarkably, a substantial fraction of those studies failed to unveil a correlation between *in vitro* and *in vivo* responses, even when less stringent parameters such as cell growth or viability instead of apoptotic death were chosen (16,17). Comparing short-term *in vitro* survival of primary samples obtained from patients diagnosed with acute myeloid leukemia with their respective clinical outcome, Möllgård and colleagues reported a significantly lower fraction of

surviving cells following exposure to daunorubicin *in vitro* in the group of patients that later achieved a complete remission after induction therapy, whereas a similar effect was missed in response to cytarabin, the probably most effective compound against this entity in the clinic (18). Patients displaying *in vitro* sensitivity to daunorubicin also showed a tendency towards better overall survival, whereas, paradoxically, patients who were *in vitro* sensitive to cytarabin suffered from a significantly reduced overall survival. The work by Rödel and colleagues is one of the sparse reports where the apoptotic capacity of a pretreatment biopsy had predictive value for the outcome of the same patient group: high-level spontaneous apoptosis assayed *in situ* in rectal cancer specimens obtained prior to neoadjuvant radiochemotherapy was associated with a significantly improved recurrence-free survival period (19). Given the poor correlation between *in vitro* chemosensitivity and patient outcome irrespective of apoptosis or mere cell viability as the primary readout, one may conclude that drug-inducible cell death simply is not a critical component of chemotherapeutic effector mechanisms *in vivo*, or, as an alternative explanation, that short-term assays fail to sufficiently mimic the complexity of drug action in a cancer patient.

3. Apoptosis and Clonogenic Survival After Drug Exposure *In Vitro*

Clonogenic survival assays are considered the gold standard to assess the interference of cytotoxic therapies with the long-term growth capacity of cancer cells *in vitro*, because they are less biased by the complicated overlap of dying and regrowing subpopulations (20). Although initiation of classic programmed cell death will force susceptible tumor cells to die within hours, attenuated forms of apoptosis may result in delayed death that can be missed by short-term viability assays, but will reduce clonogenic survival. In turn, drug-inducible arrest programs may affect clonogenic survival, because proliferation-stalled cells cannot contribute to colony formation, but may give rise to a relapse tumor when cells re-enter the cycle much later (21). Moreover, defined genetic defects in arrest-controlling checkpoints, for example in the CDK inhibitor *p21*, have been shown not to impact on clonogenic survival after γ -irradiation, although the shift from an arrest response to more apoptotic death produced a significantly higher proportion of remissions *in vivo* (22). Likewise, the comparison of cell populations differing in the expression status of the strictly antiapoptotic *bcl2* gene failed to unveil a significant difference in clonogenic outgrowth based on the apoptotic defect (23–25), suggesting a drug-inducible arrest as an alternative outcome in the presence of an apoptotic block. In addition, two other mechanisms may contribute to the inability of clonogenic assays to accurately assess the role of apoptotic defects on long-term outcome: first, to score proliferating colonies that emerged from single cells plated at extremely low densities is highly artificial and based on strong selection for mutations that facilitate growth under this extreme condition (25), and second, factors of the tumor microenvironment influencing growth *in vivo* might be completely missed in the Petri dish environment of a standard clonogenic assay *in vitro*.

4. Apoptosis and the Role of the Tumor Microenvironment

Indeed, components of the tumor microenvironment have been shown to profoundly alter clonogenic outgrowth *in vitro* (25–27). Physiological life and death decisions, for example in the germinal center of a lymph follicle, not only depend on cell autonomous

determinants, but also on prosurvival and pro-apoptotic factors of specific compartments or “niches,” and even tumor cells that harbor severe apoptotic defects are not entirely refractory to extracellular survival or death signals. Importantly, environmental signals may select for tumor cell mutations providing a growth advantage, or may “epigenetically” adjust gene activities without provoking mutations (e.g., CD154/CD40 signaling to activate the NF- κ B survival cascade [28]), hereby generating a form of nonstructural apoptotic resistance that relies on the presence of anti-apoptotic factors. Furthermore, there is increasing evidence that complex interactions between extracellular signals and cell-autonomous death mediators may be important for setting individual apoptotic thresholds: CD95 (or Fas) death receptor-triggered translocation of acid sphingomyelinase to the outer plasma membrane releases the extracellularly oriented sphingolipid ceramide, which, in turn, is essential for clustering of CD95 into lipid rafts and subsequent CD95-mediated cell death (29), and the process of C95 redistribution into lipid rafts can be augmented by DNA damaging anticancer agents such as cisplatin (30). Nevertheless, the potential crosstalk between the p53-controlled intrinsic or mitochondrial cell death pathway primarily engaged by DNA damaging compounds and death receptor mediated apoptosis still remains an issue of debate (31–33). However, there is good evidence that the combination therapy of conventional anticancer agents and proapoptotic death receptor ligands such as the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) may overcome resistance to apoptosis (34,35). Moreover, anoikis (i.e., apoptosis resulting from disruption of fibronectin-mediated cell anchorage) has been shown in an in vivo model of acute myelogenous leukemia to synergize with classic DNA damage-induced apoptosis (36). Hence, cell-autonomous and nonautonomous functions control the susceptibility of a tumor cell to undergo apoptosis in response to therapeutic stimuli.

5. Apoptosis is Important for Treatment Outcome In Vivo

Despite the concerns about the validity of clonogenic assays to predict drug responses in vivo and to serve as an appropriate model system to assess the role of apoptosis as a therapeutic effector program, there is convincing evidence from more physiological models that apoptosis indeed is important for the outcome of cancer therapy.

Matched pairs of primary, Myc-induced mouse lymphomas infected with either a mock control or stably expressing Bcl2 displayed significant differences in their responsiveness to cyclophosphamide, an alkylating anticancer agent widely used in the clinic, when growing as systemic transplant tumors at their natural tumor sites in immunocompetent mice (25,37). Moreover, long-term observation of treated mice harboring Bcl2 overexpressing vs control lymphomas unveiled a profound difference in their overall survival, demonstrating that an anti-apoptotic lesion may produce chemoresistance in vivo that was not detected by standard clonogenic assays in vitro (38). Other transgenic cancer models have been exploited to study treatment responses of tumors with defined genetic lesions as well, and demonstrated inferior outcome for syngenic tumors with p53 or *INK4a/ARF* deletions or activating *Akt* mutations (39,40), but the impact of treatment-induced apoptosis or genetic defects in apoptosis-controlling genes such as p53 on long-term responses remained uncertain when investigated in different mouse models (41–43). Besides the fact that the power of transgenic mouse models to study treatment responses based on defined genetic lesions is still underrated, most of the

mouse models used so far to analyze drug action and outcome *in vivo* are only correlative in nature. At least, overexpression of wild-type p53 in xenograft models underscored its potential to improve drug responses, albeit with no clear discrimination which of the p53-controlled effector functions actually contributed to treatment outcome (44,45). In clinical samples of chronic lymphocytic leukemia (CLL), pretreatment with alkylating agents has been shown to correlate significantly with the occurrence of p53 mutations (46), and p53 inactivation, in turn, correlates with impaired overall survival (47). Although some clinical investigations failed—possibly for technical reasons—to identify a positive correlation between p53 status and treatment outcome, many clinical trials successfully linked p53 mutations and poor outcome throughout various entities (*see ref. 48 and references therein*).

An intrinsic obstacle to scientifically address the potentially causative role of apoptosis in treatment outcome is the difficulty to generate in a biologically meaningful way isogenic primary tumors that only differ in the status of a strictly apoptosis-conferring genetic moiety relevant to drug-induced apoptosis. Matched pairs of primary tumor samples with and without a gene deletion can be generated by homologous recombination, stable expression of a small interfering RNA, a dominant-negative activity or introduction of an antiapoptotic candidate gene. Although these pairs can be used to directly assess the role of the respective moiety in treatment responses, the “addition” or “subtraction” of a genetic activity to an already established cancer cell certainly is different from a mutation acquired during tumor development. In turn, genetic lesions such as nonconditional “knockout” deletions provided during tumorigenesis may produce malignancies that indeed form in dependency on this defect, but the corresponding control tumors may not necessarily reflect a true biological “match” anymore.

6. Provoking Apoptosis in Apoptotically Compromised Tumors

Apoptotic defects are a hallmark of cancer cells (49), because successful transformation of normal cells by mitogenic oncogenes such as Myc or oncogenic Ras requires the ablation of cell-cycle checkpoint-mediated cellular failsafe mechanisms such as apoptosis (Fig. 1E). Oncogenic signaling is sensed in primary cells by the ARF/p53 axis, which, in turn, is targeted by inactivating mutations in many cancer entities (50–52). Importantly, drug-induced DNA damage is sensed and transduced into an apoptotic signal that merges with the antioncogenic signaling machinery at the level of p53. Hence, oncogene-provoked and DNA damage-induced cell death utilize pathways that, at least in part, overlap, and mutations acquired during tumor formation, for example in the apoptotic regulator genes *bax*, *bcl2*, or *Apaf-1*, may already co-select for resistance to DNA damaging therapies prior to any drug encounter (53–55).

However, the broad prevalence of apoptotic defects throughout different cancer entities is not mutually linked to clinical resistance to first line anticancer therapies. In fact, most cancer patients, even when suffering from one of the typically “chemo-insensitive” solid tumor entities, primarily respond to DNA damaging therapies and achieve clinical remissions, or, at least, a substantial reduction of their tumor burden. Of note, this clinical observation is not necessarily a lethal blow for the “apoptotic concept” which considers programmed cell death a relevant component of drug action and treatment outcome. Although apoptosis ultimately reflects the activation of a relatively small set of effector caspases and nucleases, genetic defects in these downstream players seem to

be much less frequent as compared with mutations in mediators that regulate apoptosis—and other cellular functions probably important for tumor development and possibly DNA damage signaling—more upstream. Whereas these lesions, for example at the level of p53 or the protein kinase Akt, can have profound impact on tumor development and clearly promote chemoresistance in suitable preclinical models (39,40), dose and combinatorial effects of a multiple-agent chemotherapy may initially suffice to clinically override a genetic block, but apoptotically even more severely compromised cells will rapidly re-emerge and produce a clinical relapse that is now overtly refractory not only to the initial, but also to biochemically unrelated second- and third-line therapies (56). Necrotic death, although undoubtedly provoked by anticancer agents to some extent, cannot account for the acquisition of more robustly resistant tumor cells during subsequent courses of chemotherapy. Thus, apoptosis signaling most likely reflects a well-controlled and reinforced network of interconnected mediators and modulators rather than much more vulnerable linear and unique pathways. Whereas subtle defects in response to activated oncogenes may still license tumor development, they might not fully block treatment-induced apoptosis as a consequence of supra-physiological DNA damage broadly activating multiple pro-apoptotic effector pathways. In turn, tumor-promoting and mutually resistance-conferring defects in apoptotic cellular failsafe programs can be highly specific for a certain oncogene-triggered pathway, thereby precluding tumors driven by different oncogenes to benefit from a lesion-specific resensitizing therapy (40). Furthermore, the view of apoptosis as a “network concept” also integrates the common observation of multiple apoptotic defects not only in an individual tumor specimen as a consequence of tumor heterogeneity but in a single tumor cell. Interestingly, despite their, in theory, mutually exclusive action in the same linear death pathway, pro-apoptotic mediators such as Bax and p53 can be found co-mutated, and, as such, were reported to produce a more thoroughly chemoresistant phenotype (57). Given the increasing acquisition of apoptotic defects during tumor progression and anticancer therapy, it is an attractive hypothesis that cumulative apoptotic insufficiency ultimately creates the molecular basis for clinically relevant chemoresistance.

7. Apoptogenic Therapy in Apoptosis Resistant Tumors

Using DNA damaging anticancer agents against an apoptosis-refractory tumor might be even more detrimental than just ineffective. Whereas alternative regimens based on empirically less effective compounds still produce DNA damage but little cytotoxicity, they may promote mutability and genomic instability in the surviving tumor. Patients suffering from high-frequency microsatellite instability-driven colon cancer that is based on defects in the mismatch-repair system reportedly have, in contrast with patients with sporadic, nonmismatch repair-deficiency-driven colon cancer, an inferior prognosis when exposed to adjuvant chemotherapy as compared to no treatment, implying that an otherwise apoptosis-inducing DNA damaging therapy can be harmful *per se* and may result in reduced patient survival (58). Of note, a thorough investigation of a potential mutagenic impact of DNA damaging therapies on heavily pretreated, apoptotically resistant tumors is very difficult to conduct in clinical trials, because these patients have rapidly progressive diseases for numerous reasons, and this analysis would be only correlative at best. Unfortunately, to our knowledge, there are little data from preclinical models available that addressed the role of treatment-induced DNA

damage in apoptosis-refractory tumors with respect to genomic instability, tumor progression, overall survival, and, on the other side, potentially beneficial, alternative effector programs. Indeed, as demonstrated in a transgenic mouse model, tumors that already harbor an apoptotic block can acquire mutations of genes unrelated to apoptosis during additional courses of chemotherapy, and these mutations may now interfere with non-apoptotic drug effector programs (38).

8. Senescence, Mitotic Catastrophe, and Other Programmed Cellular Responses to Therapy

It should be stressed that apoptosis is not the only programmed cellular response to anticancer therapies. Aberrant types of apoptosis (i.e., forms of programmed cell death with atypical morphological features or delayed kinetics) including mitotic catastrophe as an attenuated form of apoptosis frequently observed in response to antimicrotubule agents in p53-inactivated or spindle checkpoint-deficient cells that may endoreduplicate and become multinucleated before they eventually undergo programmed cell death (59,60), have already been discussed. The occurrence and functional relevance of these aberrant presentations of apoptosis once again underscore the view that distinct defects in checkpoint control or apoptotic execution may not necessarily prevent the ultimate outcome, but may modify its timing and biological way of execution. Importantly, severe DNA damage not only precludes a cell from further cycling by initiating programmed cell death, but may alternatively provoke an acutely inducible form of a terminal cell-cycle arrest, termed premature senescence. Various chemotherapeutic drugs have been shown to execute cellular senescence *in vitro* and *in vivo* (38,61,62), and data from a preclinical mouse model provided evidence that drug-induced senescence in apoptotically compromised tumors can improve the overall outcome to therapy (38). Whereas assessing the definite contribution of drug-inducible senescence and other programmed cellular responses to tumor biology and long-term prognosis requires further investigation, it is clear that nonapoptotic drug effector programs not only may interfere with cytotoxicity assays *in vitro*, but could also explain why so many studies failed to demonstrate a strong correlation between apoptotic capability and long-term outcome *in vivo*. The actual contribution of different components of drug action depends on many factors and cellular preconditions (i.e., cellular senescence may come into play as a backup mechanism when apoptosis is blocked). Finally, singular defects in crucial drug effector programs may not directly translate into clinical resistance, but very subtle differences in the rate of growth-eliminated cells in a certain time frame might very well determine a tumor's likelihood to reemerge as a more aggressive and more resistant relapse (56,63).

9. Provoking Apoptosis in Nonneoplastic Cells

The clinical relevance of drug-inducible apoptosis depends on the therapeutic index (i.e., the margin of tumor cell death that can be achieved by a certain dose of a compound without unacceptably harming the normal cell compartment). The underlying biological mechanisms that determine the different sensitivities to anticancer agents in malignant compared with nonneoplastic cells are not fully elucidated yet. Although the proliferation rate reflecting the requirement for accurate DNA replication and the presentation at certain cell-cycle phases in which targets of specific chemotherapeutic

agents might be particularly active undoubtedly play a role for drug efficacy, other factors seem to be important as well. Normal cells with stem cell-like properties, such as hematopoietic progenitor cells and self-renewing cells in the intestinal crypts and at the hair follicles, are prime targets for chemotherapeutic side effects, but can more easily recover from DNA damage as a result of their rather arrest- and repair-driven response compared with an apoptosis- or senescence-oriented response executed in cancer cells. Interestingly, pretreatment of mice with a small compound temporarily inhibiting p53 function was shown to decrease the extent of typical chemotherapy-related side effects such as weight loss and intestinal crypt cell apoptosis (64). Despite equal amounts of toxicity delivered to the cells, and irrespective of the possibility that cells with a high division rate may accumulate more unrepaired DNA damage, acutely transformed cells have been shown to be substantially more sensitive to apoptosis (65). This oncogene-generated gain-of-sensitivity cannot be entirely attributed to altered growth characteristics, and relies on the different utilization of growth-independent regulators such as caspases and cathepsins in apoptosis or senescence-like effector programs (66,67). Moreover, oncogenes may also sensitize to receptor-mediated cell death, as reported for the Fas-initiated death program as a component of Myc-provoked apoptosis (68).

Furthermore, drug-induced apoptosis in non-neoplastic cells might still correspond with the genetics of the adjacent tumor cells. Although p53-deficient tumors are *per se* more resistant to environmental pro-apoptotic stimuli (69), mutant p53 may promote neo-angiogenesis via enhanced secretion of factors such as the vascular endothelial growth factor (VEGF), and the rich network of tumor vessels, in turn, might protect the tumor from lethal shortage in supplies when some endothelial cells are codamaged during chemotherapy (70). Interestingly, not only p53 inactivation in the neoplastic cells may increase their apoptotic resistance to the consequences of endothelial damage (71), but cancer cells and their surrounding vasculature may even share the same resistance-conferring genetic defects if derived from a common precursor cell—an attractive, currently highly debated concept (72). Accordingly, inherited genetic preconditions to evade apoptosis in normal cells for example in patients suffering from Li-Fraumeni syndrome resulting from germline *p53* mutations or suffering from ataxia telangiectasia caused by defects in the *ATM* gene may impact on treatment outcome, but clinical data cannot distinguish treatment effects on non-neoplastic from tumor cells (73,74).

Taken together, the spatial and temporal effects of DNA damaging therapies at natural tumor sites *in vivo* are complicated to dissect and reflect a combination of direct drug action in normal and tumor cells as well as complex interactions between different cellular compartments and secreted factors. Despite an intrinsically enhanced apoptotic propensity in cancer compared to normal cells it is clear that the scientific evaluation of the actual contribution of apoptosis—and other programmed responses—to the outcome of DNA damaging anticancer therapies requires model systems that can adequately mimic the patient scenario.

10. Therapy Induced Apoptosis and Solid Cancers

Although DNA damaging therapies are widely used in the clinic against virtually all kinds of disseminated cancers or in adjuvant scenarios of many tumor entities, their long-term efficacy in solid cancer settings with few exceptions such as germ cell tumors, is vastly reduced. Whereas many hematological malignancies are potentially

curable by polychemotherapy, metastasized carcinomas, in principle, are not. Two different explanations could account for this clinically challenging problem: there is no signaling network available in epithelial cancer cells that could respond to DNA damage with some kind of acute or delayed programmed cell death, or doses applicable without producing unacceptable side effects are too low to activate an apoptotic response in these cells. To date, there is no definite answer to the underlying mechanisms of resistance in solid tumors. On one side, there are conflicting reports on the prognostic role of apoptotic defects: for example, specific *p53* mutations were shown to correlate with *de novo* resistance to doxorubicin in breast cancer patients (75), whereas low-level Bcl2 expression was associated with short overall survival in this entity (76). On the other side, high-dose chemotherapy (i.e., a relative dose escalation made possible by autologous stem cell support or as part of the conditioning regimen in allogeneic stem cell transplantation protocols) has mostly failed to improve outcome in patients with metastasized solid tumors, although other factors may interfere with outcome, especially in allogeneic transplant settings. Clinical trials based on refined strategies are required to reach more definite answers about the susceptibility to apoptosis under intensified dose regimens, which must not exceed the toxicity thresholds of non-hematopoietic organs (77,78). Unlike lymphoid cells with their inherent propensity to commit suicide during the selection of self- vs non-self-reactivity in the germinal center, or to expand in response to a certain antigen and to later regress by controlled apoptosis, most postembryonic epithelial cells do not regulate their cell number by programmed cell death, but, for example, by shedding mucosal cells into the intestinal lumen. Experimental data suggest that epithelial tumor cells die with much slower kinetics than cells from hematological malignancies, and, therefore, may rely much more on the efficacy of their DNA repair machinery, before they might undergo an attenuated form of apoptosis (20). Although carcinomas apparently select for mutations in pro-apoptotic genes during tumor development, these defects are hardly sufficient to explain the resistant behavior in response to therapy. We favor the explanation that oncogenic activation indeed provokes apoptotic defects required for tumor formation, albeit with rather modest interference with drug-related DNA damage-sensing and apoptosis-executing pathways. Although first line chemotherapies often achieve remissions against metastasized solid cancers, this potential is quickly lost in subsequent therapies, and additional DNA damage instead promotes the progression via mutability. Thus, DNA damage might be an inadequate trigger for cell death in epithelial cells, whereas other pro-apoptotic stimuli with different target specificity could have therapeutic potential.

11. Pro-Apoptotic Agents in the Clinic

The frustrating results of conventional anticancer agents in therapeutic regimens against solid tumors have prompted the development of pathogenesis-driven, lesion-specific compounds. Following the empirical strategies employed over decades for classic DNA damaging drugs, the unselected use of novel lesion-based, pro-apoptotic compounds in tumor entities irrespective of a matching genetic defect failed to produce encouraging results in various entities (79–81). For example, the orally active tyrosine kinase inhibitor gefitinib targeting the epidermal growth factor receptor (EGFR) was tested alone or in combination with standard chemotherapy in non-small cell lung

cancer patients, where it was of no therapeutic benefit (82–85). However, when patients were substratified by their molecular EGFR status, mutations in the EGFR that activate antiapoptotic pathways were found to predict responsiveness to gefitinib (86–88), and responses, at least in part, are based on the induction of apoptosis (89). Interestingly, different mutations in the EGFR have now been described in patients that had acquired resistance to gefitinib after an initial response to the compound (90). Likewise, the clinical responsiveness to the proapoptotic monoclonal antibody trastuzumab, targeting the human EGFR HER2 in breast cancer patients, was dramatically improved when patients were selected for gene amplification or overexpression of the trastuzumab target by immunohistochemistry or fluorescence *in situ* hybridization of their tumor specimens (91–94). Moreover, many of the novel mechanism-based but not lesion-based agents such as histone deacetylase inhibitors, DNA demethylating agents, or immunomodulatory thalidomide analogs interfere with complex cellular processes which may impact, in part, on programmed cell death as well. For example, the proteasome inhibitor bortezomib has pleiotropic effects on angiogenesis and tumor growth, but also blocks NF- κ B prosurvival signaling (95). Hence, proapoptotic agents can improve the clinical outcome of patients suffering from solid tumors if the compounds target cellular pathways that appear to be deregulated as components of the cell type specific transformation process.

Possibly as a result of their inherently higher apoptotic susceptibility, hematological malignancies seem to be particularly susceptible entities for proapoptotic targeted therapies. Evidence from early clinical trials suggests effectiveness of a *bcl2* antisense molecule as a single agent and in combination with conventional chemotherapy in patients from various groups of hematological malignancies (96). Given the prominent role of Bcl2 family member-governed deregulation of apoptosis in the mitochondrial pathway acquired during tumor formation or in response to chemotherapy, proapoptotic moieties that counter this mechanism could be of enormous therapeutic potential, especially in hematological malignancies with explicit translocations involving the *bcl2* gene. Moreover, a peptide-based stapled form of the BH3 death domain derived from the proapoptotic Bcl2 family member BID exerted antileukemic activity in a preclinical model *in vivo* (97). A similar approach based on peptide–peptide interactions has been employed to functionally disrupt another central regulator of cell survival in hematological malignancies: the Bcl6 oncoprotein, frequently activated in human lymphomas (98), can suppress p53 expression in the germinal center (99). Peptides that disrupt Bcl6 binding to corepressors have now been shown to cause apoptosis and cell-cycle arrest in Bcl6-positive B cell lymphomas (100) and might soon become a novel treatment option in the clinic.

12. Conclusions

Determining the role of apoptosis for the outcome of cancer therapy has been difficult. Compared with the complexity of the treatment response at a natural tumor site *in vivo*, most experimental settings fail to sufficiently mimic the tumor microenvironment and may miss components of drug action critical for long-term responses and, ultimately, for outcome *in vivo*. Tractable mouse models harboring primary tumors with defined genetic lesions and clinical evidence produced by the use of highly specific targeted therapies are expected to provide deeper and biologically more relevant insights

than conventional cell culture-based approaches. Importantly, whereas apoptosis as a programmed response to DNA damage-inducing therapies clearly interferes with treatment outcome, other programmed effector mechanisms such as premature senescence may also contribute to the overall response, because most of the currently available compounds—conventional DNA damaging chemotherapy as well as lesion-specific small molecules—cannot exclusively execute apoptosis. Likewise, most of the novel agents targeting dysfunctional processes rather than specific molecular lesions will, via a broad spectrum of effector functions, ultimately interfere with the apoptosis machinery as well. Components capable of blocking pathogenesis-related, tumor-essential pro-survival pathways contribute to clinical remissions by executing apoptosis, and this effect is not limited to hematological malignancies, but occurs in solid tumors as well. Future investigations in appropriate preclinical models and probably soon in clinical trials will elucidate whether cotargeting tumor-sensitized apoptotic signal cascades such as the TRAIL receptor-mediated death cascade may be of additional therapeutic benefit. In light of the growing arsenal of cancer-specific, apoptosis-related, and dysfunction-targeting small compounds and antibodies, the broadly DNA damaging drugs with their imprecise and devastating action will expectedly lose their clinical value as prime choice in the treatment of disseminated tumors.

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Coupling Apoptosis and Cell Division Control in Cancer

The Survivin Paradigm

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Summary

The interface between cell proliferation and cell death is thought to function as a pivotal cross-road essential to the preservation of normal homeostasis and to eliminate dangerous cells before they divide. Survivin is a prototype molecule at this crossroad, intercalated in protection against mitochondrial cell death and orchestrating various aspects of cell division. Dramatically exploited in cancer and an unfavorable gene signature for disease outcome, the survivin pathway provides tangible opportunities for targeted, rational cancer therapy.

Key Words: Survivin; apoptosis; cancer; IAP; stress response.

1. Introduction—Apoptotic Pathways

Apoptosis is generally defined as a genetic program of cellular suicide with unique morphologic characteristics (1). Essential during embryonic and fetal development (2), and for maintaining the homeostasis of differentiated tissues (3), apoptotic mechanisms are commonly subverted in cancer, where increased cell survival and/or reduced cell death are invariable molecular traits of transformed cells (4).

Among the regulators of apoptosis, Bcl-2 proteins (5) act at the mitochondria to decrease (antiapoptotic) or enhance (proapoptotic) permeability transition, typically by regulating cytochrome *c* release (3). Conversely, members of the inhibitors of apoptosis (IAP) gene family have been implicated in the regulation of a downstream step in cell survival, by preventing the activation of caspases or by inhibiting the catalytic activity of the mature enzymes. Other mechanisms of IAP-mediated cytoprotection have been also demonstrated, including ubiquitin-dependent destruction of bound caspases or mitochondrial proapoptogenic proteins (i.e., Smac) (6). The molecular signature of IAP is the presence of 1 to 3 copies of an approximate 70-amino acid zinc finger fold designated baculovirus IAP repeat (BIR), which is conserved in related molecules from yeast to humans. Certain IAPs also contain a caspase-recruitment domain (CARD), a really interesting new gene (RING) finger, a ubiquitin-conjugating domain, and a nucleotide binding P-loop motif (6). The anti-caspase activity of IAPs is evolutionary conserved, and *Drosophila* IAP-like proteins are essential regulators of cell survival in flies (7).

1.1. Survivin Structure Function

At 16.5 kDa, survivin is the smallest mammalian member of the IAP gene family (6). Structurally, it contains a single BIR, an approximate 70-amino acid zinc finger fold that

is the hallmark of all IAPs, and an extended –COOH terminus α -helical coiled-coil, but no other identifiable domain (8) typically found in IAPs. Based on X-ray crystallography of the human (9,10) or mouse (11) protein, survivin is a stable homodimer in solution with the –COOH terminus α -helices protruding from the core dimer. A single copy *survivin* gene located on chromosome 17q25 (human), or 11E2 (mouse), gives rise to four alternatively spliced survivin transcripts. In addition to wild-type survivin (142 amino acids), three survivin isoforms are generated by insertion of an alternative exon 2 (survivin-2B, 165 amino acids), removal of exon 3 (survivin- Δ Ex-3, 137 amino acids) (14), or acquisition of an in-frame stop codon in intron 2 (Survivin-2 α) (15). In survivin- Δ Ex-3, the splicing event introduces a frame shift that generates a unique –COOH terminus of potential functional significance (16).

A unique property of survivin is a sharp cell cycle-dependent expression at mitosis. This is largely, but not exclusively, controlled at the level of gene transcription and involves canonical CDE/CHR boxes in the survivin promoter (17,18), acting as potential G1-repressor elements. Among the post-translational modifications that affect survivin levels, interest has recently focused on the control of protein stability. Survivin is a relatively short-lived protein ($t^{1/2}$ = 30 min), and polyubiquitylation followed by proteasomal destruction has been shown to contribute to cell-cycle periodicity by keeping survivin levels low at interphase (19). In addition, mitotic phosphorylation of survivin on Thr34 by p34cdc2–cyclin B1 has been associated with increased survivin stability at metaphase (20).

There are also examples of modulation of survivin expression independently of cell-cycle progression. For CD34⁺ bone-marrow-derived stem cells, stimulation with hematopoietic cytokines resulted in survivin expression in the absence of cyclin D and with hypophosphorylated Rb (21), and vascular remodeling by angiopoietin-1 (Ang-1) upregulated survivin in nonproliferating endothelial cells (22,23). The search for signaling intermediates that control survivin expression has generated interesting findings. One common denominator of this pathway is the requirement for PI3-kinase/Akt activation, a general antiapoptotic signal (24) that has been implicated in up-regulation of survivin induced by granulocyte macrophage-colony stimulating factor (GM-CSF), CSF, Ang-1, and fibronectin-dependent cell adhesion (21,22,25,26). In addition, modulation of survivin expression has been observed in endothelial cells stimulated with nonmitogenic concentrations of interleukin (IL)-11 (27), engagement of vascular endothelial (VE)-cadherin expression, and function (28), or angiotensin II stimulation (29). Another critical transcriptional regulator of survivin expression in pleural effusion lymphoma cell sustained by vascular endothelial growth factor (VEGF), IL-6, or IL-10 (30) was identified as activated signal transducers and activators of transcription (STAT)3. This is potentially important for the known role of STAT3 in cytoprotection (31), which may have direct consequences for oncogenic transformation (32).

2. Role of Survivin in Cell Division

Upon expression at mitosis, survivin localizes to various components of the mitotic apparatus, including centrosomes, microtubules of the metaphase and anaphase spindle, and the remnants of the mitotic apparatus—midbodies (12,33–36). A direct association between survivin and polymerized tubulin has been demonstrated in vitro (12), potentially involving the –COOH terminus α -helices. A subcellular pool of survivin has also been localized to kinetochores of metaphase chromosomes (35,37), and may comprise

up to about 20% of total cellular survivin (36). On the other hand, survivin is largely excluded from the nucleus (36) through a CRM1-dependent mechanism that may involve the survivin –COOH terminus (38). Survivin is also a mitotic phosphoprotein. In addition to phosphorylation on Thr34 by the main mitotic kinase p34cdc2, phosphorylation of survivin on Thr117 by Aurora B kinase has been also reported (39) as potentially important in regulating the topography and/or association of the chromosomal passenger complex (discussed later).

The complex localization of survivin to the mitotic apparatus reflects an essential function at cell division. This first surfaced in targeting experiments using antisense or dominant-negative mutants, which resulted in a dual phenotype of apoptosis (discussed later) and aberrant mitotic progression, with supernumerary centrosomes, multipolar mitotic spindles, failed cytokinesis, and multinucleation (33). Parallel experiments targeting survivin-like molecules in yeast (40) or *Caenorhabditis elegans* (41) also revealed lethal cell division defects with failure to assemble a cleavage furrow and inability to complete cytokinesis. The phenotype of survivin knockout mice is consistent with an essential role of this pathway at mitosis (42). Beginning at embryonic day (E) 2.5, homozygous deletion of the *survivin* gene resulted in catastrophic defects of microtubule assembly, with absence of mitotic spindles, complete failure of cell division and multinucleation with 100% lethality by E3.5 to 4.5 (42). An identical phenotype was independently confirmed on three different mouse genetic backgrounds (43). Therefore, one model that has been put forward is that survivin functions in an essential and evolutionary conserved step in late stage cell division (i.e., cytokinesis, potentially involving cleavage furrow formation [42,44]). In support of this, survivin was shown to form a complex with molecules comprising the so-called “chromosomal passenger complex,” including Aurora B kinase and INCENP on kinetochores and the anaphase central spindle (37,45), and to enhance the activity of Aurora B kinase in both mammalian cells (46) and *Xenopus laevis* (47). However, recent data suggest that survivin may play a broader role at various aspects of cell division. This point became apparent in antibody microinjection studies, which revealed a composite phenotype of prolonged metaphase arrest, occasionally followed by apoptosis, formation of multipolar mitotic spindles, and defects in chromosome attachments (48,49). By immunofluorescence, cells microinjected with an antibody to survivin exhibited flattened and abortive mitotic spindles severely depleted of microtubules (49). Conversely, retroviral or adenoviral expression of survivin restored spindle stability and dynamics against microtubule poisons in tumor cells (49) as well as endothelial cells (50). A critical role of survivin in microtubule function and spindle formation was also demonstrated using RNAi ablation of survivin expression in nontransformed human fibroblasts (51). This is consistent with a number of other investigations using survivin knockdown by RNA interference. In these experiments, loss of survivin expression was associated with inability to complete mitosis, stable prometaphase arrest, defective function of the spindle assembly checkpoint, and aberrant spindle formation (52–54). Altogether, these data point to a more general role of survivin at cell division, and not exclusively limited to cytokinesis.

This possibility has recently gained further credibility from a more in-depth analysis of the chromosomal passenger complex. Different from earlier suggestions that implicated the chromosomal passenger complex solely in kinetochore attachment and cytokinesis, recent evidence has uncovered a key role of these molecules in spindle

formation via enhanced microtubule stabilization (55,56). Whether the proposed association of survivin with Aurora B kinase is directly involved in this mechanism remains to be seen, but it is clear that a large subcellular pool of mitotic survivin is not in complex with the chromosomal passenger complex (36). This suggests that the chromosomal passenger complex and survivin may exert potentially independent roles in microtubule function and mitotic spindle assembly. Such a model is consistent with the observation that microtubule- and kinetochore-associated survivin are differentially regulated during the cell cycle, exhibit nonoverlapping patterns of phosphorylation, and are recognized by monoclonal antibodies (MAbs) in a mutually exclusive fashion (36). As an alternative possibility, it cannot be ruled out that the differently spliced survivin isoforms could mediate independent functions at cell division (14). This remains a possibility that requires formal testing when antibodies capable of discriminating between the various endogenous survivin isoforms become available. However, wild-type survivin is clearly the overwhelming form in all cell types tested thus far (14), and, at least in over-expression experiments, survivin-2B does not associate with the mitotic apparatus, and survivin-ΔEx-3 has actually been localized to the nucleus potentially through a bipartite nuclear localization signal in the new –COOH terminus (16,38).

In sum, it appears likely that the various survivin pools and/or alternatively spliced variants of survivin provide a continuum of functions throughout mitosis. This may involve regulation of centrosomal activity, the assembly of a competent bipolar mitotic spindles, the control of kinetochore-microtubule attachment, and cytokinesis. This model is also satisfactory if one considers the biology of Aurora kinases, which, like survivin, are found frequently over-expressed in human cancers, and may participate in oncogenic transformation. Altogether, the combined over-expression of both survivin and Aurora in cancer may obliterate the surveillance mechanism of the spindle assembly checkpoint, allowing cells with spindle defects, aberrant chromosome congression, or misaligned/unattached kinetochores to proceed through cell division, thus enhancing tumor-associated aneuploidy.

3. Role of Survivin in Apoptosis Inhibition

The question as to whether survivin had also a distinct and separable role in apoptosis inhibition, similarly to other mammalian IAPs (6), or whether its function is limited to cell division, as with IAPs in yeast and *C. elegans* (40,41), has been amply investigated in recent years. With the expansion of experimental work on survivin, three lines of experimental evidence clearly support the notion that survivin has an independent and separable role in apoptosis inhibition, in vitro and in vivo.

First, over-expression of survivin has been associated with inhibition of cell death initiated via the extrinsic or intrinsic apoptotic pathways (57–60). Second, transgenic expression of survivin resulted in apoptosis inhibition in vivo (61), and livers isolated from heterozygous survivin^{+/-} animals exhibited exaggerated apoptosis in response to suboptimal ligation of Fas (43). Third, molecular antagonists of survivin, including antisense, ribozymes, siRNA sequences, or dominant-negative mutants resulted in caspase-dependent cell death, enhancement of apoptotic stimuli, and anticancer activity, in vivo (57–60). The apoptosis inhibitory function of survivin is evolutionary conserved, and a survivin-like molecule in *Drosophila*, *Deterin*, functions interchangeably with survivin to block apoptosis (62) in mammalian or insect cells (34).

Although the notion that survivin inhibits apoptosis is well established, the more mechanistic aspects of this pathway have long remained elusive. As with other IAPs (6), a physical interaction between survivin and initiator or effector caspases has been reported by several groups (17,63,64), and a physical proximity between survivin and effector caspase-3 has been seen in vivo as well (33). However, with the exception of two published studies (65,66), this did not seem to translate in meaningful inhibition of caspase activity, in vitro (9,64,67). In addition, the crystal structure of survivin does not suggest the presence of a “hook-and-sinker” region that mediates caspase binding in other IAPs (9), and the residues in the linker upstream BIR2 that in other IAPs (i.e., X-linked mammalian inhibitor of apoptosis protein [XIAP]) dock to caspase-3 (68), are not present in survivin. One can take the view that this does not automatically rule out that survivin may still function as an inhibitor of effector caspases similarly to other IAPs (6), and arguments to explain the lack of activity in cell-free systems may include folding requirements of the recombinant protein (66), or the need for additional protein partners, in vivo (discussed later).

On the other hand, evidence is accumulating that survivin may play a more selective role than other IAPs in antagonizing mitochondrial-dependent apoptosis. Overexpression of survivin is more efficient at blocking mitochondrial- but not death-receptor-induced apoptosis (61), a complex between survivin and the upstream mitochondrial initiator caspase-9 has been demonstrated in vivo (69), and survivin has been shown to associate with Smac/DIABLO (70–73), a mitochondrial-released apoptogenic protein that relieves the inhibitory effect of IAPs on caspase activation (68). Moreover, cell death induced by molecular antagonists of survivin or by heterozygous reduction of survivin levels (43) has the characteristics of mitochondrial-dependent apoptosis with cytochrome *c* release (74), caspase-9 activation (69), and involvement of the apoptosome components (20), caspase-9 and Apaf-1 (75). Collectively, these data suggest that survivin, differently from other IAPs that inhibit initiator or effector caspases through their independent BIRs (68), may selectively target the multimolecular process of caspase-9 activation.

Although an attractive candidate, this pathway does not seem to be centered on the recognition of Smac/DIABLO by survivin (76). Although this complex has been recently characterized in detail (73), its role in regulating survivin-dependent cytoprotection has not been firmly demonstrated (70,72). Conversely, recent experimental evidence provided unexpected clues to explain the pathway of survivin cytoprotection and its relevance to tumor progression, in vivo. In subcellular fractionation experiments, a novel and abundant pool of survivin was recently localized to mitochondria, and further mapped biochemically to the intermitochondrial membrane space (77). Mitochondrial survivin was shown to function as a highly dynamic subcellular pool, enriched in response to cellular stress and promptly discharged in the cytosol following exposure to a variety of cell death agonists (77). In turn, mitochondrially-released survivin was competent to inhibit the upstream initiation of mitochondrial apoptosis, prevent processing and activation of apoptosome-associated caspase-9, and enhance tumor growth in immunocompromised animals (77). Conversely, survivin that could not be transported to mitochondria had no role in apoptosis inhibition and did not provide any advantage for accelerated tumor growth, in vivo. From these studies (78), it is clear that the function of survivin in apoptosis inhibition is separable from its second role at mitosis, and is specifically mediated by a discrete subcellular (mitochondrial) pool of the

molecule. In broader terms, these data also demonstrate that mitochondrial permeability transition results not only in the release of proapoptogenic mediators but also of at least one apoptosis inhibitor, potentially ideally positioned to counter-balance the apoptosome initiation of cell death (78). Further studies are needed to clarify why survivin must travel to mitochondria and be dynamically discharged in the cytosol in order to become competent to inhibit apoptosis. However, given the complexity and functional relevance of survivin posttranslational modifications, it seems plausible to hypothesize that mitochondrial or cytosolic survivin undergo different and mutually exclusive posttranslational modifications that either enable or prevent its role in blocking apoptosis. Another critical aspect of the pathway is that mitochondrially released survivin acts in the cytosol to prevent apoptosis. This may reflect the mechanistic requirement of functional cofactors needed to perform as a high affinity apoptosis inhibitor. In this context, survivin has been shown to associate with cytosolic proteins, including HBXIP (79), and another IAP family protein, XIAP (80), and that formation of these complexes results in enhanced survivin anti-apoptotic activity.

A critical role of survivin in mitochondrial apoptosis is also consistent with a similar function ascribed to one of its splice variant, survivin- Δ Ex3. It was recently reported that survivin- Δ Ex3 contains a mitochondrial localization signal and a BH2 domain in the new, alternatively spliced -COOH terminus (81), and that a survivin- Δ Ex3-like molecule in the herpes simplex virus (HSV) genome localized to mitochondria and inhibited apoptosis by associating with Bcl-2 and by suppressing caspase-3 activity through its BIR (81). Whether survivin- Δ Ex3 behaves similarly to its viral counterpart remains to be seen, but its potential import to mitochondria would make it ideally suited to act, alone or by dimerizing with wild type survivin, as an upstream regulator of mitochondrial-dependent apoptosis.

4. Translational Targeting of the Survivin Pathway in Cancer

One of the most significant features of survivin is its differential expression in cancer vs normal tissues (8). Reminiscent of “onco-fetal” antigens, survivin is strongly expressed in embryonic and fetal organs (17,82), undetectable or found at very low levels in most terminally differentiated normal tissues (8), and dramatically over-expressed in most human cancers surveyed (83). The selective over-expression of survivin in cancer appears to reflect a global deregulation of *survivin* gene transcription in transformed cells. This is consistent with the cancer-specific transcription of the *survivin* promoter (84), and the several oncogenic pathways that converge to up-regulate *survivin* gene expression in transformed cells. These include growth factor receptor signaling (85,86), STAT activation (30), PI3 kinase/Akt signaling (87), oncogene (Ras) expression (88), and loss of tumor suppressor molecules, p53 (89,90), adenomatous polyposis coli (APC) (91,92), and promyelocytic leukemia (PML) (93).

Several retrospective studies have demonstrated that survivin expression in cancer is frequently associated with unfavorable disease outcome, abbreviated overall survival, increased rates of recurrences, resistance to therapy, and reduced apoptotic index, in vivo (83). Accordingly, profiling studies identified survivin as a “risk-associated” gene signature for unfavorable outcome in breast cancer (94), large cell non-Hodgkin’s lymphoma (95), and colorectal cancer (96,97). Importantly, survivin was incorporated as one of the critical 16 genes predictors of recurrences in tamoxifen-treated node-negative breast cancer patients (98).

Because of its differential expression in cancer and critical roles in mitotic progression and cell viability, survivin has been vigorously pursued as a new rational target for cancer treatment (83). Three approaches have shown promise in both preclinical and early clinical testing. First, survivin has been used for novel cancer vaccination protocols (99). Several studies have demonstrated that T-cells mount a vigorous cytolytic and antibody response to survivin peptides, in vitro and in vivo (100–106). Accordingly, HLA Class I-restricted cytolytic T-cells against survivin peptides are found in cancer patients (103,105), and exert strong antitumor activity when tested in preclinical models (107–109). Similar promising results were obtained with a survivin-directed DNA cancer vaccine that was shown to exert anticancer activity in tumor models in vivo by both affecting the tumor cell population as well as survivin-expressing angiogenic endothelial cells (110). Several phase I trials using targeted cancer immunotherapy with survivin are currently ongoing. Available published data are encouraging (111–113), and demonstrate that cancer immunotherapy with survivin generates antigen-specific immunological response in a high proportion of treated patients and, importantly, this occurs without appreciable toxicity for normal tissues and organs. Altogether, these observations warrant further expansion of survivin cancer immunotherapy approaches in larger phase II trials.

Secondly, molecular antagonists of survivin, including antisense, ribozymes, siRNA, or dominant negative mutants have shown reproducible antitumor efficacy in vitro and in a variety of tumor models, in vivo. These have been consistently associated with enhanced tumor cell apoptosis, suppression of cell proliferation, and collapse of tumor-associated angiogenesis (97,114–119). With respect to advanced clinical development, a phase I trial using survivin antisense oligonucleotides has recently been completed.

Thirdly, molecular (74) or pharmacological (20) antagonists of survivin phosphorylation on Thr34 have also shown promising preclinical results in suppression of tumor growth in mice, and enhancement of taxane-based chemotherapy (20). In particular, sequential inhibition of p34cdc2 kinase activity by small molecule antagonists following mitotic arrest imposed by taxanes resulted in loss of survivin levels, massive induction of apoptosis, and inhibition of tumor growth in mouse models (20,120).

A novel aspect of survivin cytoprotection in cancer has recently emerged with its link to the molecular chaperone Hsp90 (121). Heat shock proteins (Hsps), in particular Hsp90 and Hsp70 are molecular chaperones that survey protein folding quality control, and play an essential role in the cellular stress response in cancer (122), thus enabling transformed cells to constantly adapt to unfavorable environmental challenges (123). Binding to Hsp90 preserves survivin levels in cells and disruption of this interaction results in proteasomal degradation of survivin and a dual phenotype of spontaneous apoptosis and mitotic defects (121). Although at a much earlier stage of development compared to other approaches, selective disruption of the survivin-Hsp90 interaction could be envisioned as an alternative strategy to destabilize survivin levels in tumor cells, leading to suppression of cell proliferation and initiation of mitochondrial apoptosis (121).

5. Conclusions

Despite its relatively recent discovery in 1997, survivin has attracted considerable attention from several viewpoints of biomedical sciences. In particular, its dramatic overexpression in virtually every human cancer coupled with its dual function in cell

proliferation and cell survival and its clinical link to unfavorable disease outcome have made survivin an attractive target for rational cancer therapy. Importantly, survivin appears essential for tumor-cell maintenance and molecular or pharmacologic interference with the survivin pathway have been consistently associated with tumor cell death and strong anticancer activity, either alone or in combination with standard cytotoxics in several preclinical models. Importantly, no evidence of tumor resistance for survivin-based therapeutic strategies has been reported. Combined with the differential expression of this pathway in tumors as opposed to normal tissues, this inspires confidence that survivin-directed therapeutics may provide a novel class of targeted agents for rational cancer treatment in humans.

Acknowledgments

This work was supported by NIH grants HL54131, CA78810 and CA90917.

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Clinical Significance of Histone Deacetylase Inhibitors in Cancer

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Summary

Chromatin remodeling agents modulate gene expression in tumor cells. Acetylation and deacetylation are catalyzed by specific enzyme families, histone acetyltransferases (HATs) and deacetylases (HDACs), respectively. Aberrant acetylation of histone and nonhistone proteins has been linked to malignant diseases. HDAC inhibitors bear great potential as new drugs because of their ability to modulate transcription, induce differentiation and apoptosis, and inhibit angiogenesis. Furthermore, HDAC inhibitors also enhance the activity of other cancer therapeutics such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), chemotherapeutic drugs, and radiotherapy. Some of the HDAC inhibitors are currently under clinical investigations. This chapter reviews the chemistry and the biology of HDACs and HDAC inhibitors, laying particular emphasis on those agents which have potentials for cancer therapy.

Key Words: HDAC inhibitors; SAHA; MS-275; TSA; TRAIL; apoptosis; caspase.

1. Introduction

Epigenetic modifications, mainly DNA methylation and acetylation, are recognized as additional mechanisms contributing to the malignant phenotype (1,2). Acetylation and deacetylation of histones plays a role in the regulation of gene expression (3). Histone acetylation is a reversible process whereby histone acetyltransferase (HAT) transfers the acetyl moiety from acetyl coenzyme A to the lysine, and histone deacetylase (HDAC) removes the acetyl groups, re-establishing the positive charge in the histones. HATs and HDACs have recently been shown to regulate cell proliferation, differentiation, and apoptosis in various hematological and solid malignancies (4,5). Altered HAT or HDAC activity is associated with cancer by changing the expression pattern of selected genes (6,7). Hyperacetylation of histones H3 and H4 correlates with gene activation, whereas deacetylation mediates eukaryotic chromatin condensation and gene expression silencing (8,9). Recently, new roles of histone acetylation have been uncovered, not only in transcription but also in DNA replication, repair, and heterochromatin formation (10).

2. Histone Deacetylases

There are at least 12 human HDAC enzymes, HDAC1-11 (11–15) and HDAC-A (16). Based on the structural properties, HDACs can be divided into three classes (17). Class I members (HDAC 1, 2, 3, 8, and 11) are transcriptional corepressors homologous to yeast RPD3 and have a single deacetylase domain at the N termini and diversified C-terminal regions (18). Class II members (HDAC 4, 5, 6, 7, 9, and 10) have domains

similar to yeast HDA1 with a deacetylase domain at a more C-terminal position (19). In addition, HDAC 6 contains a second N-terminal deacetylase domain, which can function independently of its C-terminal counterpart. Class III histone deacetylases are distinct from class I and II HDACs and are homologous of the yeast silent information regulator 2 (Sir2). All of these HDACs apparently exist in the cell as subunits of multi-protein complexes. Class II HDACs have been shown to translocate from the cytoplasm to the nucleus in response to external stimuli, whereas class I HDACs are constitutively nuclear and play important roles in dynamic gene regulation (20).

Sir2, a heterochromatin component in yeast, silences transcription at silent mating loci, telomerase, and ribosomal DNA (rDNA), and this also suppresses recombination in rDNA. Earlier experiments have shown that the overexpression of Sir2 in yeast induced the global deacetylation of histones, indicating that Sir2 was a histone deacetylase (21). Later, it was shown that *cobB*, a bacterial homolog of Sir2, had ribosyltransferase activity, leading to experiments showing that Sir2 was also able to transfer ADP-ribose from NAD (22). Subsequently, it was confirmed that Sir2 was an NAD-dependent histone deacetylase (23). The ADP-ribosylation of an acetylated lysine residue is an intermediate state of the enzymatic reaction catalyzed by Sir2. Only class III enzymes use NAD as a cofactor. Thus, these enzymes are known as NAD-dependent histone deacetylases.

Recently, Sir2 has attracted much attention, because it is related to longevity (24). The overexpression of Sir2 extends the lifespan of budding yeast, whereas its knockout shortens the lifespan by about 50% (25). Sir2 (also known as sirtuins) is conserved from bacteria to humans. In the nematodes, the gene most homologous to yeast Sir2 gene is *sir-2.1*. A duplication containing the *sir-2.1* gene confers a lifespan that is extended by up to 50% (26). The mammalian homologs consist of seven members, SIRT1–SIRT7. In mammalian cells, SIRT1 down-regulates stress-induced p53 and FOXO pathways for apoptosis, thus favoring survival under stress. In the absence of applied stress, SIRT1 silencing induces growth arrest and/or apoptosis in human epithelial cancer cells (27). In contrast, normal human epithelial cells and normal human diploid fibroblasts seem to be refractory to SIRT1 silencing. Further studies have revealed that the SIRT1-regulated pathway is independent of p53, Bax, and caspase-2. Alternatively, SIRT1 may suppress apoptosis downstream from these apoptotic factors. FOXO4 (but not FOXO3) is required as proapoptotic mediator. Caspase-3 and caspase-7 act as downstream executioners of SIRT1/FOXO4-regulated apoptosis. These data suggest that SIRT1 as a novel target for selective killing of cancer vs noncancer epithelial cells. Upregulation of SIRT1 may be a double-edged sword that both promotes survival of aging cells and increases cancer risk in mammals.

Histones are part of the core proteins of nucleosomes. The recruitment of HATs and HDACs plays an important role in proliferation, differentiation and apoptosis (4,5). Altered HAT or HDAC activity is associated with the development of cancer by changing the expression of several genes (6,7). Hyperacetylation of the N-terminal tails of histones H3 and H4 correlates with gene activation, whereas deacetylation mediates transcriptional repression (8). Treatment of malignant cells with HDAC inhibitors regulates only a small number (1–2%) of genes, as examined by DNA microarray studies (28). HDAC1 interacts directly with other transcription repressors, including all three of the pocket proteins, Rb, p107, p130, and YY1. HDAC1 causes transcription repression

by locally deacetylating histones, leading to a compact nucleosomal structure that prevents transcription factors from accessing DNA to promote transcription. Furthermore, HDAC1 knockout mice were embryonic lethal, possibly resulting from a proliferative defect upon unrestricted expressions of the cell cycle inhibitors p21^{WAF1/CIP1} and p27^{KIP1} (29). Overexpression of histone deacetylase I confers resistance to sodium butyrate-mediated apoptosis in melanoma cells through a p53-mediated pathway (30). We and others have shown that inhibition of HDAC activity induces apoptosis in various types of cancer (31–35).

Stability of HDACs is an important factor in determining the biological activity. HDAC4 is unusually unstable, with a half-life of less than 8 h (36). Consistent with the instability of HDAC4 protein, its mRNA was also highly unstable (with a half-life of less than 4 h). The exposure of cells to ultraviolet irradiation resulted in the degradation of HDAC4. This degradation was not dependent on proteasome or CRM1-mediated export activity but instead was caspase-dependent and was detectable in diverse human cancer lines. Of two potential caspase consensus motifs in HDAC4, both lying within a region containing proline, glutamic acid-, serine-, and threonine-rich (PEST) sequences, Asp-289 as the prime cleavage site was identified by site-directed mutagenesis (36). Notably, this residue is not conserved among other class IIa members, HDAC5, -7, and -9. Finally, the induced expression of caspase-cleavable HDAC4 led to markedly increased apoptosis. These results therefore link the regulation of HDAC4 protein stability to caspases, enzymes that are important for controlling cell death and differentiation.

HDACs are important regulators of gene expression as part of transcriptional corepressor complexes. Caspases can repress the activity of the myocyte enhancer factor (MEF)2C transcription factor by regulating HDAC4 processing (37). Cleavage of HDAC4 occurs at Asp 289 and disjoins the carboxy-terminal fragment, localized into the cytoplasm, from the amino-terminal fragment, which accumulates into the nucleus. In the nucleus, the caspase-generated fragment of HDAC4 is able to trigger cytochrome *c* release from mitochondria and cell death in a caspase-9-dependent manner. The caspase-cleaved amino-terminal fragment of HDAC4 acts as a strong repressor of the transcription factor MEF2C, independently from the HDAC domain. Removal of amino acids 166–289 from the caspase-cleaved fragment of HDAC4 abrogates its ability to repress MEF2 transcription and to induce cell death.

3. Histone Deacetylase Inhibitors

In transcriptionally active chromatin, histones generally are hyperacetylated and, conversely, hypoacetylated histones are coincident with silenced chromatin. Revived interest in these enzymatic pathways and how they modulate eukaryotic transcription has led to the identification of multiple cofactors whose complex interplay with HDAC affects gene expression. Concurrent with these discoveries, screening of natural product sources yielded new small molecules that were subsequently identified as potent inhibitors of HDAC. Whereas predominantly identified using antiproliferative assays, the biological activity of these new HDAC inhibitors also encompasses significant antiprotozoal, antifungal, phytotoxic, and antiviral applications. During the past decade, a number of HDAC inhibitors have been shown to induce growth arrest, differentiation, and/or apoptosis in cancer cells (31,33,38–40), and inhibit tumor growth in various xenograft models (41–48). Recently, attention has been focused on the ability of HDAC inhibitors to

induce perturbations in cell cycle regulatory proteins (e.g., p21^{WAF1/CIP1}), down regulation of survival signaling pathways (e.g., Raf/MAPkinase/ERK), and disruption of cellular redox state (e.g., reactive oxygen species [ROS]). Therefore, HDAC inhibitors are considered as candidate drugs in cancer therapy (49–51). Five classes of HDAC inhibitors have been characterized and include benzamides (i.e., MS-275); hydroxamic acids (i.e., suberolanilide hydroxamic acid and trichostatin A); short-chain fatty acids (i.e., sodium butyrate and phenylbutyrate); cyclic tetrapeptide containing a 2-amino-8-oxo-9, 10-epoxy-decanoyl moiety (i.e., trapoxin A); and cyclic peptides without the 2-amino-8-oxo-9, 10-epoxy-decanoyl moiety (i.e., FK228). A wide variety of HDAC inhibitors of both natural and synthetic origin has been reported. Except depsispeptide/FK228, natural HDACs (trichostatin [TSA]), depudecin, trapoxins, apicidins) as well as sodium butyrate, phenylbutyrate and suberoyl anilide hydroxamic acid (SAHA), while effective in vivo, are inefficient due to instability and low retention. Subsequently, synthetic analogs isolated from screening libraries (oxamflatin, scriptaid) were discovered as having a common structure with TSA and SAHA: a hydroxamic acid zinc-binding group linked via a spacer (5 or 6 CH₂) to a hydrophobic group. Design of a second generation of HDACs was based upon these data affording potent HDACs such as LAQ824 and PDX101 currently under phase I clinical trials. Simultaneously, synthetic benzamide-containing HDAC inhibitors were reported and two of them, MS-275 and CI-994, have reached phase II and I clinical trials, respectively.

3.1. Benzamides

Several benzamides have been found to inhibit HDAC activity in the low micromolar range. A 2'-hydroxy or amino function seems to be essential for the optimum activity (52). The 2'-amino compound MS-275 is the first HDAC inhibitor discovered with oral anticancer activity in an animal model. It appears to be nontoxic in several animal models. Pretreatment of human leukemic cells with MS-275 significantly enhances the abrogative capacity of an established nucleoside analogue, fludarabine (53). The study indicates that apart from promoting acetylation of histones and regulation of genes involved in differentiation and apoptosis, MS-275 also induces multiple perturbations in signal transduction, survival, and cell-cycle regulatory pathways that increase the fludarabine-mediated cell death. MS-275, acetyldinaline, and CI-994 are in clinical trials for the treatment of cancers (54,55).

3.2. Trichostatin A and Analogs

Trichostatin A (TSA) from *Streptomyces hygroscopicus* was initially identified as an antifungal agent (56), but later discovered that it induces histone hyperacetylation and terminal differentiation (57). It acts as a noncompetitive inhibitor of HDAC by mimicking the lysine substrate as well as chelating a zinc atom crucial for enzymatic activity. TSA inhibits proliferation, causes cell-cycle arrest, and induces differentiation and/or apoptosis in numerous models of leukemia, lymphoma, multiple myeloma, and solid tumors (33,54,58–69). TSA is also effective in xenograft models (70,71). TSA attenuated the development of allergic airway inflammation by decreasing expression of the Th2 cytokines, interleukin (IL)-4, and IL-5, and IgE, which resulted from reduced T-cell infiltration, suggesting that HDAC inhibition may attenuate the development of asthma by a T-cell suppressive effect (72). Other analogs of TSA such as oxamflatin, scriptaid,

and amide derivatives have been reported to have anticancer activity (54,73–75). Scriptaid induces reticulocytosis and human gamma-globin synthesis (76), suggesting a potential for scriptaid as a treatment option for sickle cell disease.

3.3. Cyclic Tetrapeptides

Hydrophobic cyclotetrapeptides contain common amino acid (S)-2-Amino-9,10-epoxy-8-xodecanoic acid (L-Aoe) and have been reported to inhibit HDACs (77,78). The epoxyketone was first thought to be essential for activity, as reduction or nucleophilic attack resulted in inactivation of compounds (77,78). Trapoxin A, a microbially derived cyclotetrapeptide, is an irreversible inhibitor in the low nanomolar range (77). Trapoxin inhibits histone deacetylation in vivo and causes mammalian cells to arrest in the cell cycle (11). On the other hand, related HC toxin (host-selective toxin of *Cochliobolus carbonum*) inhibits maize enzyme activity reversibly (78). K-trap (an analogous of trapoxin A) inhibited HDAC1 activity. A novel fungal metabolite, apicidin [cyclo(N-O-methyl-L-tryptophanyl-L-isoleuciny-D-pipecolinyl-L-2-amino-8-oxo-decanoyl)], exhibits potent, broad spectrum antiprotozoal activity in vitro against Apicomplexan parasites (79). Apicidin's antiparasitic activity appears to result from low nanomolar inhibition of HDAC, which induces hyperacetylation of histones in treated parasites. Because apicidin and apicidin A possess only a ketone functional group and are active in the low nanomolar concentrations, it appears that the presence of the epoxy group is not essential for activity. Apicidin induces differentiation, cell-cycle arrest, and apoptosis, and inhibits metastasis and angiogenesis in several cancer models (38,80–87). A number of derivatives such as 9-acyloxyapicidins or 9-hydroxy have been prepared and are under investigation. Trapoxin analogs that combine cyclotetrapeptide and hydroxamic acid moieties have been prepared. The inhibitors of quinolone analogs and the hydroxamic acid analogs of apicidin yielded promising results (88,89). Depudecin, a natural epoxide derivative isolated from the fungus *A. brassicicola*, induces hyperacetylation of histones and morphological reversion in v-ras transformed NIH 3T3 cells (90).

3.4. Tetrapeptide Analogs

Simple analogs of cyclic tetrapeptides that contains suberic acid linkers and hydroxamate, instead of epoxyketone or ketone functional group, inhibit HDAC activity (91). The structurally related hybrid polar compounds (HPC) was shown to induce differentiation in a wide variety of transformed cells (92). The first representative was hexamethylene bisacetamide (HMBA) which induced differentiation of transformed cells in millimolar range (93). HMBA regulates genes that control G1-to-S phase transition, leading to G1 arrest and inhibition of DNA synthesis. Among the inducer-mediated changes, suppression of cyclin-dependent kinase cdk4, which may be required for phosphorylation of the retinoblastoma protein pRB and perhaps p107, is critical in the pathway of terminal differentiation. HMBA induces an increase in the level of p21^{WAF1/CIP1} which inhibits cyclin-dependent kinase activity and, in turn, may cause cells to arrest in G1. p107 complexes with transcription factor E2F, which may alter E2F-dependent gene transcription. HMBA has also been shown to induce differentiation of neoplastic cells in patients. Furthermore, a second generation of hybrid polar compounds have been synthesized which are up to 1000-fold more potent than HMBA on a molar basis

as inducers of murine erythroleukemia (MEL) cells and other transformed cells in vitro. Second-generation HPCs such as suberoylanilide hydroxamic acid (SAHA), suberic bishydroxamic acid (SBHA), and m-carboxycinnamic acid bishydroxamide (CBHA) inhibited HDAC activity and induced cancer cell differentiation (69,94). SAHA has been shown to act as a chemopreventive agent in mammary tumors in the rat (95) and inhibited the growth of established tumors (41,95,96). Polyaminohydroxamic acids (PAHAs) represent an important new chemical class of HDAC inhibitors and appear to be more specific than SAHA, TSA, and MS-275, because it is selectively directed to chromatin and associated histones by the positively charged polyamine side chain. Several other analogs of hydroxamic acids are being developed (91,97).

3.5. Depsipeptide and Thiols

The depsipeptide (FR901228/FK228) induces differentiation, growth arrest, and apoptosis in the low-nanomolar range (98–109). It also inhibits metastasis and angiogenesis (108–110). Depsipeptide is also very promising antitumor agent against osteosarcoma, inducing apoptosis by the activation of the Fas/FasL system (111). Depsipeptide induced apoptosis in multiple myeloma U266 and RPMI 8226 cell lines in a time- and dose-dependent fashion, and in primary patient myeloma cells (100). Depsipeptide promotes histone acetylation, gene transcription, apoptosis and its activity is enhanced by DNA methyltransferase inhibitors in AML1/ETO-positive leukemic cells (100). Preclinical studies with depsipeptide in chronic lymphocytic leukemia (CLL) and acute myeloid leukemia (AML) have demonstrated that it effectively induces apoptosis at concentrations at which HDAC inhibition occurs. A dose-dependent increase in H3 and H4 histone acetylation was noted in depsipeptide-treated AML1/ETO-positive Kasumi-1 cells and blasts from a patient with t(8;21) AML (105). A phase 1 and pharmacodynamic study of depsipeptide in CLL and AML have yielded promising results (112).

Opening of the disulfide bridge leads to a thiol that may be able to enter the active site and complex the zinc ion. In this regard, garlic constituents and their metabolites, such as diallylsulfide and allylmercaptan, inhibited HDAC activity. Diallyl disulfide caused increased acetylation of H3 and H4 histones in DS19 mouse erythroleukemic cells and K562 human leukemic cells (113), suggesting that differentiation in erythroleukemic cells by diallyl disulfide and allyl mercaptan may be mediated through induction of histone acetylation. Acetylation was also induced in rat hepatoma and human breast cancer cells by diallyl disulfide or its metabolite, allyl mercaptan. Diallyl disulfide increased histone acetylation and p21^{WAF1/CIP1} expression in human colon tumor cell lines (114).

3.6. Psammaplins

Psammaplins, isolated from a marine sponge *Pseudoceratina purpurea*, inhibited histone deacetylase and DNA methyltransferase activities (115). Psammaplin A (PsA) contains an α -oximatoamide functional group, which inhibits the HDAC activity at the catalytic site. The disulfide group is also an essential feature for HDAC inhibition. PsA showed a potent cytotoxicity against several cancer and endothelial cells (116–123). PsA-induced cytotoxicity may correlate with its inhibition on DNA replication (116). Furthermore, PsA was found to inhibit mammalian aminopeptidase N (APN) that plays

a key role in tumor cell invasion and angiogenesis (117). Interestingly, the anti-proliferative effect of PsA was dependent on the cellular amount of APN expression. PsA suppressed the invasion and tube formation of endothelial cells stimulated by basic fibroblast growth factor. These findings suggest that PsA can be developed as antiangiogenic and anticancer agent. Several synthetic analogs of PsA are being developed.

4. Intracellular Mechanisms of HDAC Inhibitors

Recent studies have shown that HDAC inhibitors induce cell-cycle arrest, differentiation, and apoptosis in vitro and in vivo (33,34,44,61,107,111,124–134). The mechanisms by which these inhibitors induce cell cycle arrest, differentiation, and apoptosis appear to involve multiple genes. In addition to inducing growth arrest and apoptosis, they also inhibit metastasis and angiogenesis (68,87,107,135–138).

Molecular inhibition of the ErbB signaling pathway represents a promising cancer treatment strategy. Preclinical studies suggest that enhancement of antitumor activity can be achieved by maximizing ErbB signaling inhibition. It has been demonstrated that HDAC inhibitors decreased transcript expression of ErbB1 (epidermal growth factor receptor) and ErbB2 in DU145 (prostate) and ErbB2 in SKBr3 (breast) cancer cell lines (139). HDAC inhibitors also inhibited caveolin-1 and HIF- α (hypoxia inducible factor 1-alpha), and upregulated gelsolin, p19 (INK4D) and Nur77 expressions in DU145 cells (139). Enhanced proliferative inhibition, apoptosis induction, and signaling inhibition were demonstrated when combining HDAC inhibition with ErbB blockade. These results suggest that used cooperatively, anti-ErbB agents and HDAC inhibitors may offer a promising strategy of dual targeted therapy. The beneficial interaction of these agents may not derive solely from modulation of ErbB expression, but may result from effects on other oncogenic processes including angiogenesis, invasion and cell-cycle kinetics.

4.1. Effects of HDAC Inhibitors on Cell Cycle

During the cell-division cycle, chromosomal DNA must initially be precisely duplicated and then correctly segregated to daughter cells. Cell-cycle control of transcription seems to be a universal feature of proliferating cells, although relatively little is known about its biological significance and conservation between organisms.

Given the key role of cell-cycle integrity in tumor suppression and cancer therapy, a lot of attention has focused on the ability of HDAC inhibitors to alter the levels of cell-cycle regulatory proteins. HDAC inhibitors induce growth arrest at both the G1 and G2/M phases of cell cycle and induce differentiation and/or apoptosis of various types of tumor cell lines (33,34,45,69,102,126,127,130,132,140–145). HDAC inhibitors induced both p21^{WAF1/CIP1} and p27^{KIP1} at protein levels, and caused hypophosphorylation of Rb (33,69,132,146). Other cell-cycle inhibitors that participate in the proliferative arrest elicited by HDAC inhibitors are p15^{INK4b}, p18^{INK4c}, and p19^{INK4d} (147,148). Moreover, positive regulators of proliferation, such as cyclins D1 and D2, cMyc, or c-Src, are downregulated by HDAC inhibitors (149–154). P53 is activated both by inhibitors of HDACs class I/II, as well as, by inhibitors of the Sir2 family (155–158). Transcription factor Sp1 regulates p21^{WAF1/CIP1} expression in a p53-independent fashion (85,106,159,160). p21^{WAF1/CIP1} expression is also transcriptionally regulated by p53 (161).

4.2. Effects of HDAC Inhibitors on Apoptosis

HDAC inhibitors induce apoptosis in breast, prostate, lung and thyroid carcinoma, and leukemia and multiple myeloma (18,31,33,45,130,146,162–169). In addition to the induction of death receptors by HDAC inhibitors, the regulation of Bcl-2 family members is also important for inducing sensitivity (31,33,100,170–173). We and others have shown that HDAC inhibitors selectively induce proapoptotic members such as Bax, Bak, Noxa, Bim, and PUMA and inhibit antiapoptotic Mcl-1, Bcl-X_L and Bcl-2 expression. Bcl-2 family members mainly exert their apoptotic effects by acting at the level of mitochondria and play crucial role in cancer development (174). HDAC inhibitors cleave poly(ADP-ribose) polymerase (PARP) and caspase-8, -9, -3, -7, and -2. Transfection of Bcl-2 cDNA partially suppressed SAHA-induced cell death. HDAC inhibitors can also induce TRAIL, suggesting the activation of death receptor pathway without the requirement of exogenous TRAIL. Thus, HDAC inhibitors can induce apoptosis by linking both death receptor and mitochondrial pathways of apoptosis.

Dysregulation in apoptosis has been associated with the development of cancer (175). Recent studies have shown the involvement of mitochondria in many apoptotic signaling pathways (176,177). Members of the Bcl-2 family of proteins that regulate apoptotic signaling through mitochondria are key regulators of apoptosis in mammalian development, and their deregulation is associated with disease, particularly cancer (178,179). There are three classes of Bcl-2 family members: apoptosis promoters (e.g., Bax and Bak); apoptosis inhibitors (e.g., Bcl-2, Bcl-X_L, and adenoviral E1B 19K); and the BH3-only Bcl-2 family members (e.g., Bid, Puma, Noxa, Bad, and Nbk/Bik), that contain the BH3 interaction domain that act as apoptosis promoters and inhibitors (180). Therefore, the regulation of Bcl-2 family members by HDAC inhibitor may play important roles on apoptosis by inducing a death activity or by antagonizing a survival activity. Inactivation of both Bax and Bak was required for tumor growth and was selected for in vivo during tumorigenesis (181,182). Bax^{-/-} and Bak^{-/-} DKO MEFs were resistant to death signaling pathway, indicating that they are the required downstream components of mitochondrial signaling pathways (177). The induction of Bim by HDAC inhibitors in leukemia cells is similar to other study where withdrawal of cytokines from survival factor-dependent lymphocyte cell lines results in an up-regulation of Bim expression, concomitant with the induction of apoptotic program. Bim has been implicated in modulating lymphocyte homeostasis in immune cells. Bim^{-/-} mice succumb to autoimmune kidney disease, accumulation of lymphoid and myeloid cells and perturbed T-cell development (183,184).

The inhibitor of apoptosis (IAP) proteins serve as key regulators of caspase activity (185). IAPs (cIAP-1, cIAP-2, NIAP, Livin/ML-IAP, survivin, and XIAP) protect cells against apoptosis by acting as caspase inhibitors (185). IAPs bind to and directly inhibit caspase-3, -7, and -9 (185,186). IAP proteins are regulated by interactions with the mitochondrial proteins (e.g., Smac/DIABLO), which may be released into the cytosol upon apoptotic stimulation and through IAP sequestration results in elevated caspase activity (187,188). Some IAP proteins are also regulated by proteolysis via the ubiquitin-proteasome pathway and caspase-dependent cleavage of XIAP in cells undergoing apoptosis. The inhibition of XIAP, cIAP1, and cIAP2 expressions by HDAC inhibitors may contribute in sensitization of cells to TRAIL. In this context, we have shown that TRAIL inhibits the expression of IAPs in breast and prostate cancer cells (31,69). The

combination of HDAC inhibitors and TRAIL may further inhibit the expression of some of the IAPs and contribute to the synergistic induction of apoptosis by these agents.

HDAC inhibitors activate the p53 molecule through acetylation of 320 and 373 lysine residues, upregulate PIG3 and NOXA and induce apoptosis in cancer cells expressing wild and pseudo-wild type p53 genes (189).

SAHA induced polyploidy in human colon cancer cell line HCT116 and human breast cancer cell lines, MCF-7, MDA-MB-231, and MBA-MD-468, but not in normal human embryonic fibroblast SW-38 and normal mouse embryonic fibroblasts (190). The polyploid cells lost the capacity for proliferation and committed to senescence. The induction of polyploidy was enhanced in HCT116 p21^{WAF1-/-} or HCT116 p^{53-/-} cells than in wild-type HCT116. The development of senescence of SAHA-induced polyploidy cells was similar in all colon cell lines (191). The present findings indicate that the HDAC inhibitor could exert antitumor effects by inducing polyploidy, and this effect is more marked in transformed cells with nonfunctioning p21^{WAF1/CIP1} or p53 genes.

In chronic myelocytic leukemia (CML) the activity of the Bcr-Abl tyrosine kinase is known to activate a number of molecular mechanisms, which inhibit apoptosis (191,192). SAHA markedly decreases protein expression levels of Bcr-Abl and c-Myc and HDAC3 in CML, suggesting that SAHA exerts its biological activity by inhibiting survival pathway (191). Differential expression of histone deacetylase has been reported in various cancer. To explore the mechanisms of disease-specific HDAC activity in acute myeloid leukaemia (AML), the expression of the HDAC in primary AML blasts and in four control cell types, namely CD34⁺ progenitors from umbilical cord, either quiescent or cycling (postculture), cycling CD34⁺ progenitors from GCSF-stimulated adult donors and peripheral blood mononuclear cells (PBMCs) was characterized. Only SIRT1 was consistently overexpressed in AML samples compared with all controls, while HDAC6 was overexpressed relative to adult, but not neonatal cells (193). HDAC5 and SIRT4 were consistently underexpressed. HDAC inhibitors (valproate, butyrate, TSA, SAHA) caused hyperacetylation of histones in AML blasts and cell lines (193). Such treatment also modulated the pattern of HDAC expression, with strong induction of HDAC11 in all myeloid cells tested, and lesser, more selective, induction of HDAC9 and SIRT4. The distinct pattern of HDAC expression in AML and its response to HDAC inhibitors is of relevance to the development of HDAC inhibitor-based therapeutic strategies and may contribute to observed patterns of clinical response and development of drug resistance.

4.3. Effects of HDAC Inhibitors on Angiogenesis

Tumor growth requires the development of new vessels that sprout from pre-existing normal vessels in a process known as “angiogenesis” (194). These new vessels arise from local capillaries, arteries, and veins in response to the release of soluble growth factors from the tumor mass, enabling these tumors to grow beyond the diffusion-limited size of approx 2-mm diameter. Tumor growth and metastasis depend upon the development of a neovasculature in and around the tumor (194–199). Angiogenesis is regulated by the balance between stimulatory (e.g., basic fibroblast growth factor [bFGF], IL-8, matrix metalloproteinase [MMP]-2, MMP-9, tumor growth factor [TGF]β1, vascular endothelial growth factor [VEGF]) and inhibitory (e.g., angiostatin,

IL-10, interferon) factors released by the tumor and its environment (196,200). For example, overexpression of bFGF (201,202) and VEGF (203–205) has been found in the tissue, serum, and urine of patients with bladder cancer and has been associated with cancer progression, suggesting a direct involvement of these proteins in angiogenesis.

HDAC inhibitors also inhibit endothelial cell proliferation and angiogenesis by downregulating angiogenesis-related gene expression (44,68,87,107–109,135–139,206–219). TSA has been reported to inhibit angiogenesis both in vitro and in vivo (68). TSA inhibits hypoxia-induced production of the angiogenic mediator VEGF by tumor cells and also inhibits directly endothelial cell migration and proliferation (68). These data suggest that HDAC inhibitors can inhibit tumor growth by inhibiting angiogenesis, and their effects on angiogenesis can be further enhanced in the presence of TRAIL.

The combination of adenoviral vector carrying wild type p53 (Ad-p53) gene therapy with sodium butyrate resulted in a complete regression of xenografted human gastric tumor (KATO-III) cells in nude mice (44). Tumors treated with the combination showed higher numbers of terminal dUTP nick end labeling (TUNEL) positive cells and lower CD34 staining than those treated with a single modality (44). This was further supported by the finding that BAI-1 (brain specific angiogenesis inhibitor-1), an inhibitor of vascularization, was induced by sodium butyrate treatment in cells transfected with Ad-p53 (44). These data suggest that HDAC inhibitors can be combined with p53 gene therapy for the treatment of cancer. The finding that HDAC appears to be a critical regulator of angiogenesis in addition to tumor cell growth will heighten interest in the development of HDAC inhibitors as potential antiangiogenic and anticancer drugs.

5. HDAC Inhibitors and TRAIL/Apo-2L

We and other have shown that several HDAC inhibitors can enhance the apoptosis-inducing potential of TRAIL and sensitize TRAIL-resistant breast, prostate, and lung cancer cells, and malignant mesothelioma, leukemia, and myeloma cells (31,33,98,162,220–224). The sensitization of TRAIL resistant cells appears to result from downregulation of the antiapoptotic protein Bcl-2, Bcl-X_L, Mcl-1, and upregulation of proapoptotic genes Bax, Bak, TRAIL, Fas, FasL, DR4, and DR5, and activation of caspases. HDAC inhibitors upregulate proapoptotic genes only in cancer cells but not in normal cells (225,226). Sodium butyrate and TSA enhanced TRAIL-mediated apoptosis to a greater extent than decapeptide, MS-275 and oxamflatin (98). Both sodium butyrate and TSA treatment also increased mRNA and surface expression of TRAIL-R2/DR5 that was dependent on the transcription factor Sp1, thus providing a possible mechanism behind the increased sensitivity to TRAIL. These results show that sensitivity to HDAC inhibitors in cancer cells is a property of the fully transformed phenotype and depends on activation of a specific death pathway. Because HDAC inhibitors sensitize TRAIL-resistant cancer cells to undergo apoptosis by TRAIL, they appear to be promising candidates for combination chemotherapy.

Several studies have demonstrated the engagement of mitochondria during activation of death receptor pathway (69,227–229). Crosstalk between the death-receptor (extrinsic) and mitochondrial (intrinsic) pathways requires caspase-8/-10 dependent cleavage of Bid (31,33,69,227). tBid activates Bax and Bak to release cytochrome *c* and other mitochondrial proteins (176,230). Because HDAC inhibitors induced cleavage of Bid, the truncated Bid may trigger activation of mitochondria in the absence of ligand

TRAIL. We have shown that the pan-caspase inhibitor z-VAD-fmk completely inhibited TRAIL-induced apoptosis in the presence of HDAC inhibitor (31,33,69). The caspase-8 inhibitor z-IETD and DN-FADD completely inhibited the synergistic interaction between HDAC inhibitor and TRAIL. Furthermore, in the presence of HDAC inhibitors, TRAIL induced caspase-3 and caspase-9 activation and caused cleavage of their substrate PARP. Antiapoptotic proteins Bcl-2 and Bcl-X_L inhibit HDAC inhibitors and/or TRAIL-induced apoptosis by blocking cytochrome *c* release. The phosphorylation deficient mutant of Bcl-2 and Bcl-X_L also inhibited HDAC inhibitors and/or TRAIL-induced apoptosis. In cell-intrinsic pathway of apoptosis, mitochondria amplify the apoptotic signals leading to activation of caspase-9 (177). Caspase-9 in turn activates downstream caspases and the cleavage of apoptotic substrates that finally kill cells. The synergistic effects of HDAC inhibitors and TRAIL on apoptosis occur through activation of downstream caspase-3, which can be activated by both extrinsic and intrinsic pathways (31,33,69).

The sensitization of cancer cells to HDAC inhibitors appears to be p53 independent. We have recently shown that chemotherapeutic drugs (231) or irradiation (232,233) can sensitize breast and prostate cancer cells by upregulating death receptors DR4 and/or DR5 in cells harboring wild-type (MCF-7) and mutated (MDA-MB-231, and MDA-MB-468) p53. Recent studies have shown that HDAC inhibitors induce apoptosis in leukemia in a p53-independent manner but not in normal hematopoietic progenitors (220,225). Other transcription factors such as NFκB and SP1 have been shown to regulate the expression of death receptors (234–237).

Treatment of nude mice with HDAC inhibitors resulted in acetylation of histone H3 and H4, and down-regulation of hypoxia-inducible factor 1-α and VEGF expression in tumor cells. Furthermore, control mice demonstrating increased rate of tumor growth had increased numbers of CD31-positive or von Willebrand Factor (vWF)-positive blood vessels, and increased circulating vascular VEGFR2-positive endothelial cells compared with HDAC inhibitor and/or TRAIL treated mice. Sequential treatments of athymic nude mice with HDAC inhibitors followed by TRAIL cause a synergistic apoptotic response through activation of caspase-3 and caspase-7, which is accompanied by regression of tumor growth, inhibition of angiogenesis and enhancement of survival of xenografted nude mice. Together with our previous studies showing that cancer chemotherapeutic drugs and irradiation up-regulate DR4 and/or DR5 expression, thereby enhancing TRAIL-induced apoptosis in vivo (231,233,238,239), these studies demonstrate the antitumor interactions of HDAC inhibitors with the TRAIL-death receptor pathway. Similarly, several recent studies including ours have demonstrated the additive or synergistic effects of HDAC inhibitors and TRAIL on apoptosis in vitro (31,33,69,162,170,171,220–224). The ability of HDAC inhibitors to sensitize cancer cells to TRAIL suggests that HDAC inhibitors can reduce the minimal effective dose or side effects of TRAIL. Thus, these data provide the framework for clinical evaluation of HDAC inhibitors and TRAIL for the treatment of human cancer.

6. HDAC Inhibitors and Irradiation

HDAC inhibitors have been shown to radiosensitize breast, prostate, and glioma cells lines (132,240,241). TSA has been shown to radiosensitize human glioblastoma U373MG and U87MG cell lines in a dose- and time-dependent manner (240). Valproic acid

enhanced the radiosensitivity of brain tumor SF539 and U251 cell lines in vitro and U251 xenografts in vivo, which correlated with the induction of histone hyperacetylation (242). Similarly, MS-275 can enhance radiosensitivity of DU145 prostate carcinoma and U251 glioma cells suggesting that this effect may involve an inhibition of DNA repair (241). The combination of HDAC inhibitors with irradiation may be a useful for the treatment of cancer and merit further investigation. Given the limited efficacy of standard treatments for patients with cancer, these data provide support for clinical trials integrating HDAC inhibitor with radiation therapy.

Caspase-2 and caspase-3 cleave HDAC4 in vitro and caspase-3 is critical for HDAC4 cleavage in vivo during UV-induced apoptosis (37). After ultraviolet irradiation, GFP-HDAC4 translocates into the nucleus coincidentally/immediately before the retraction response, but clearly before nuclear fragmentation. Together, these data indicate that caspases could specifically modulate gene repression and apoptosis through the proteolytic processing of HDAC4. Among molecular cell cycle-targeted drugs currently in the pipeline for testing in early-phase clinical trials, HDAC inhibitors may have therapeutic potential as radiosensitizers.

7. HDAC Inhibitors and Chemotherapeutic Drugs

Chemotherapeutic treatment with combinations of drugs is front-line therapy for many types of cancer. Combining drugs which target different signaling pathways often lessens adverse side effects while increasing the efficacy of treatment and reducing patient morbidity. It has recently been shown that HDAC inhibitors facilitate the cytotoxic effectiveness of the topoisomerase I inhibitor camptothecin in the killing of tumor cells (243). HDAC inhibitors (SAHA and sodium butyrate) interacted synergistically with camptothecin in inducing apoptosis of breast and lung cancer cell lines. Experiments have shown that cells arrested in G2-M by camptothecin were most sensitive to subsequent addition of HDAC inhibitor. In camptothecin-arrested cells, sodium butyrate decreases cyclin B levels, as well as the levels of the antiapoptotic proteins XIAP and survivin. Overall, these findings suggest that reducing the levels of these critical antiapoptotic factors may increase the efficacy of camptothecin in the clinical setting if given in a sequence that does not prevent or inhibit tumor cell progression through the S phase.

MS-275 also synergistically interacted with fludarabine in inducing apoptosis of lymphoid and myeloid human leukemia cells (53). Prior exposure of Jurkat lymphoblastic leukemia cells to MS-275 increased mitochondrial injury, caspase activation, and apoptosis in response to fludarabine, resulting in highly synergistic antileukemic interactions and loss of clonogenic survival. Simultaneous exposure to MS-275 and fludarabine also led to synergistic effects, but these were not as pronounced as observed with sequential treatment. Similar interactions were noted in the case of (1) other human leukemia cell lines (e.g., U937, CCRF-CEM); (2) other HDAC inhibitors (e.g., sodium butyrate); and (3) other nucleoside analogues (e.g., 1- β -D-arabinofuranosylcytosine, gemcitabine). Potentiation of fludarabine-induced apoptosis by MS-275 was associated with acetylation of histones H3 and H4, down-regulation of the antiapoptotic proteins XIAP and Mcl-1, enhanced cytosolic release of proapoptotic mitochondrial proteins (e.g., cytochrome *c*, Smac/DIABLO, and AIF), and caspase activation. These events were accompanied by the caspase-dependent down-regulation of p27^{KIP1}, cyclins A, E,

and D1, and cleavage and diminished phosphorylation of retinoblastoma protein. Prior exposure to MS-275 attenuated fludarabine-mediated activation of MEK1/2, extracellular signal-regulated kinase, and Akt, and enhanced c-Jun NH(2)-terminal kinase phosphorylation; furthermore, inducible expression of constitutively active MEK1/2 or Akt significantly diminished MS-275/fludarabine-induced lethality. Combined exposure of cells to MS-275 and fludarabine was associated with a significant increase in generation of ROS; moreover, both the increase in ROS and apoptosis were largely attenuated by coadministration of the free radical scavenger L-N-acetylcysteine. Finally, prior administration of MS-275 markedly potentiated fludarabine-mediated generation of the proapoptotic lipid second messenger ceramide. Taken together, these findings indicate that MS-275 induces multiple perturbations in signal transduction, survival, and cell cycle regulatory pathways that lower the threshold for fludarabine-mediated mitochondrial injury and apoptosis in human leukemia cells.

A synergistic interaction of retinoic acid and CBHA was shown in a mouse model of neuroblastoma. DNA hypomethylating agents have been found to have synergistic effects with HDAC inhibitors. The combination of TSA with azacytidine caused a dramatic potentiation in the activation of silenced genes (244–246). Dipeptide and TSA induced apoptosis in human lung cancer cells, and HDAC inhibitor-induced apoptosis was greatly enhanced in the presence of the DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (DAC), suggesting the DNA methylation status plays an important role on the effectiveness of HDAC inhibitors (247). Furthermore, HDAC inhibitors enhanced paclitaxel-induced cell death in ovarian cancer cell lines independent of p53 status (248). Similarly, commonly used anticancer drugs doxorubicin and decitabine have been reported to have synergistic effects with HDAC inhibitors (249,250). Thus, the combination of anticancer drugs with other epigenetic therapies provides potentially safer therapeutic options.

8. HDAC Inhibitors and Chemopreventive Drugs

In recent years, the use of naturally occurring chemopreventive agents have attracted many investigators because of their nontoxic effects. The preclinical data on selected chemopreventive agents have been very promising. Sulforaphane (SFN), an isothiocyanate first isolated from broccoli, exhibits chemopreventive properties in prostate cancer cells through mechanisms that are poorly understood. SFN inhibits HDAC activity in colon and prostate cancer cells (251). The inhibition of HDAC was accompanied by an increase in acetylated histones. SFN caused enhanced interaction of acetylated histone H4 with the promoter region of the p²¹/WAF1/CIP1 gene and the bax gene. A corresponding 1.5- to 2-fold increase was seen for p²¹/WAF1/CIP1 and Bax protein expression, consistent with previous studies using HDAC inhibitors such as trichostatin A. SFN induced cell-cycle arrest and apoptosis through caspase activation. These findings provide new insight into the mechanisms of SFN action in benign prostate hyperplasia, and they suggest a novel approach to chemoprotection and chemotherapy of prostate cancer through the inhibition of HDAC.

9. Clinical Trials With HDAC Inhibitors

Initial clinical trials indicate that HDAC inhibitors from several different structural classes are very well tolerated and exhibit clinical activity against a variety of human

malignancies; however, the molecular basis for their anticancer selectivity remains largely unknown. HDAC inhibitors have also shown preclinical promise when combined with other therapeutic agents, and innovative drug delivery strategies, including liposome encapsulation, may further enhance their clinical development and anticancer potential. An improved understanding of the mechanistic role of specific HDACs in human tumorigenesis, as well as the identification of more specific HDAC inhibitors, will likely accelerate the clinical development and broaden the future scope and utility of HDAC inhibitors for cancer treatment.

Several HDAC inhibitors (SAHA, MS-275, CI-994 and depsipeptide) are currently undergoing clinical trials (252–254). HDAC inhibitors represent a relatively new group of targeted anticancer compounds, which are showing significant promise as agents with activity against a broad spectrum of neoplasms, at doses that are well tolerated by cancer patients. SAHA is one of the HDAC inhibitors most advanced in development. It is in phase I and II clinical trials for patients with both hematologic and solid tumors (252). Phase I/II clinical trials on depsipeptide have shown low toxicity and evidence of antitumor activity; on the other hand, this compound has potential for synergism with radiotherapy, chemotherapy, and biologicals. Second generation HDAC inhibitors such as LAQ824 and PDX101 are currently under phase I clinical trials. Simultaneously, synthetic benzamide-containing HDACs were reported and two of them, MS-275 and CI-994, have reached phase II and I clinical trials, respectively.

10. Conclusions

The histone deacetylase has been considered a target molecule for cancer therapy. The inhibition of HDAC activity by a specific inhibitor induces growth arrest, differentiation, and apoptosis of transformed or several cancer cells. The discovery and development of specific HDAC inhibitors are helpful for cancer therapy, and decipher the molecular mode of action for HDAC.

Given that HDAC inhibitors upregulate proapoptotic members TRAIL-R1/DR4, TRAIL-R2/DR5, Bax, Bak, PUMA, and NOXA and downregulate antiapoptotic Bcl-2, Bcl-X_L and Mcl-1 proteins, it is possible that sensitization of cancer cells to chemotherapy, irradiation, or TRAIL by HDAC inhibitors may occur at various stages of apoptotic pathways. Furthermore, the ability of HDAC inhibitors to inhibit angiogenesis may further affect tumor growth by regulating angiogenesis-related signaling pathways. Preliminary studies in animal models have revealed a relatively high tumor selectivity of HDAC inhibitors, strengthening their promising potential in cancer chemotherapy. Some of these inhibitors are in a clinical trial at phase I or phase II. Furthermore, the combination of HDAC inhibitors with commonly used anticancer drugs, irradiation or TRAIL will be useful for cancer therapy.

Acknowledgment

This work was supported by grants from the Susan G. Komen Breast Cancer Foundation.

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