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APOPTOSIS IN TUMORIGENESIS AND CANCER THERAPY

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TABLE OF CONTENTS

1. ABSTRACT

Apoptosis is a morphologically and biochemically distinct form of cell death which can be triggered by a variety of extracellular agents during both normal development as well as in adult pathological states. Much progress has recently been made in understanding the molecular pathways which regulate this process as well as new intersections between these. A direct interaction between components of the 'executioner' - the ICE-family of cysteine proteases - and the Bcl-2 family of proteins, which modulate a cell's propensity to undergo apoptosis, has recently been demonstrated. New pathways to cell survival, like the PI3-K/Akt signal transduction pathway, are also providing new clues as to the regulation of cell death by growth factors and extracellular matrix for example. The links which exist between apoptosis and cancer research are several. Genetic alterations in components of the apoptosis pathway occur during tumorigenesis and confer resistance to a variety of physiological (oncogene-induced cell death, loss of adhesion, growth under hypoxia) as well as therapeutic (chemotherapy and radiation) death triggers. Similarly, antineoplastic therapies are thought to induce tumor cell apoptosis, and consequently, common mutations in apoptosis-regulatory genes carry a poor prognosis for the patient. A more detailed understanding of the biochemistry of apoptosis and the ways in which it is disabled in tumors will likely reveal new transformation selective death triggers which stimulate cell death in ways independent of components like p53 and increase the therapeutic window of these drugs in the clinics.

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2. INTRODUCTION

The study of apoptosis is relevant to many aspects of tumor biology which include tumorigenesis, tumor homeostasis, angiogenesis, metastasis, and clinical treatment. Malignant cells often harbor mutations in critical components of the apoptotic pathway which may correlate with poor prognosis. Solid tumors must circumvent the apoptotic pathways which regulate their anchorage-dependent survival in order to metastasize and establish secondary tumor sites. Although chemotherapy was long thought to kill tumor cells by inactivating critical metabolic pathways, it is now recognized that many effective chemotherapeutics trigger the tumor into killing itself by activation of an apoptotic pathway. It is therefore of prime importance for the discovery of improved treatments to better understand the molecular underpinnings of the apoptosis machinery. It is crucial to remember, however, that much of the knowledge we have gained has been derived from the study of 'developmental apoptosis' - or programmed cell death - in primitive organisms amenable to genetic manipulation. Given the highly conserved nature of the apoptosis machinery (at least parts of it), uncovering its molecular details using a variety of *in vitro* and genetic systems will likely provide useful information for cancer therapy.

Apoptosis is a morphologically and biochemically distinct form of cell death which can be likened to 'cellular suicide.' The apoptotic cell actively destroys itself in a manner which will neither harm neighboring cells nor induce an inflammatory response. This physiological process is distinct from necrotic cell death, which occurs as a result of severe cell injury and results in swelling and lysis. The genetically programmed death of cells during normal development is sometimes referred to as "programmed cell death," an example of apoptosis in normal physiology. During the development of most, if not all, multicellular organisms, programmed cell death provides an efficient mechanism whereby unwanted cells are eliminated. Its relevance has been documented in a number of instances which include thymocyte maturation, formation of the (wrongly named) inter digital "necrotic zones" during limb morphogenesis, mammary gland involution, spermatogonial and follicular development, and synaptogenesis during neural development among others. Whereas the death triggers specific for each of these systems are as diverse as the systems themselves, common apoptosis "effector pathways" appear to be conserved throughout evolution from nematodes to vertebrates. Pathways leading to and responsible for this widespread phenomenon have been incessantly populated with new trigger, effector, and inhibitor molecules. Accrued interest in deciphering these pathways has, in part, been sparked by the recent understanding that successful antineoplastic therapies may also operate through induction of tumor cell-specific apoptosis.

This review focuses on aspects of apoptosis research which have gained particular attention in recent years. Advances in the 'executioner' machinery, its substrates, the Bcl-2 family, Fas-induced death, and the PI3-K/Akt pathway are described with particular focus on the use of genetic and *in vitro* systems to identify new participants. We subsequently provide an overview of the relevance of these topics in understanding issues specific to tumor biology such as tumorigenesis, anchorage-independent survival and metastasis, tumor-induced angiogenesis, and chemotherapy.

3. THE BIOCHEMISTRY OF APOPTOSIS

3.1 The executioner and its substrates

Our molecular understanding of apoptosis has advanced profoundly since its original description by Wyllie and colleagues (1). While the first hallmarks of apoptotic cell death - membrane blebbing, chromatin condensation, and cellular fragmentation into 'apoptotic bodies' - were purely morphological, biochemical hallmarks have superseded these. Apoptosis is now studied as a cascade of proteases and endonucleases, where oligonucleosomal DNA laddering and cleavage of a variety of substrates by cysteine proteases have become the modern "gold-standards." Our knowledge of the executioner as well as some of its key modulators stems in large part from genetic studies of the nematode *C. elegans*.

Apoptosis is a developmentally programmed process in *C. elegans* whereby the death of individual cells is genetically determined and reproducibly observed. Mutants in which this pattern of programmed death is disturbed were described in the Horvitz laboratory at M.I.T., which led to the discovery of the cell death (*ced*) genes. Nematode mutants in the ced genes displayed a variety of phenotypes ranging from defects in engulfment by neighboring cells to excess death or survival (2, 3). In particular, the *ced-3* and *ced-4* genes were found to be required for the death of the normally occuring 131 programmed cell death events during *C. elegans* development. Gain-of-function mutations in the *ced-9* gene suggested it may function to modulate the activity of *ced-3* and *ced-4* (4). Cloning of Interleukin 1-beta Converting Enzyme (ICE) protease as a mammalian homologue for CED-3 provided the first indication that proteases may play a critical role in apoptosis (5, 6). Following this observation, numerous ICE-family proteases have been identified in mammalian cells and are thought to constitute the core of the apoptosis executioner.

The ICE proteases all belong to the cysteine protease subfamily characterized by a cysteine residue at the active site. ICE/ced-3 homologues contain the conserved QACRG sequence surrounding the catalytic cysteine and show a preference for cleaving substrates after aspartate residues. Phylogenetic relationships among the proteases has led to their subdivision into three families (7). Typically, these enzymes can autocatalytically cleave and activate themselves as well as other ICE-family proteases. This may lead to amplification and diversification of available substrates during the execution phase of apoptosis in cells. The specific amino-acid sequence N-terminal to the target aspartate determines substrate preference among proteases of the ICE family: CPP32-like proteases cleave following DEVD, while ICE-like proteases cleave following YVAD. Specific peptide inhibitors which mimic cellular substrates containing these sequences have proved useful in studying the role and timing of activation of these proteases both in cells and *in vitro*. In the attempt to simplify the nomenclature for this ever-expanding family of enzymes, apoptosis proteases have recently been renamed 'caspases' (or *c*ysteine proteases which cleave after *asp*artic acid), and numbered in function of the chronology of their discovery (8).

The ICE-family proteases known to date (over 10 members in mammals), strongly suggest that, unlike in *C. elegans* or other lower organisms, there may be considerable functional overlap within the death machinery of higher species. Alternatively, the wide variety of target substrates discovered so far in addition to *in vivo* observations afforded by the generation of knock-out mice, suggest that some proteases may have

organ/tissue-specific expression patterns and/or cleave specific substrates within the cell. Mice deficient for CPP32, for example, display a brain-restricted apoptosis defect reminiscent of the neuron-specific phenotype of nematodes lacking *ced-3* (9). Mice defective for ICE - the prototype of the apoptosis cysteine proteases - show no significant apoptosis defects during development or in response to apoptosis triggers such as ionizing radiation or dexamethazone, but are resistant to Fas-induced death (10, 11). While both these genetic studies point to the restricted tissue/pathway function of individual proteases in apoptosis, more extensive knock-out studies and crosses should reveal whether this trend holds true for other ICE-homologues.

One of the central questions remains how proteolysis leads to the demise of the cell and its stereotypical changes. It is still unclear which substrates known to date (if any) are instrumental in the death pathway and which are simply biochemical markers of the process. Poly ADP-ribose polymerase (PARP) was one of the first proteins reported to be cleaved during apoptosis (12, 13), and is a target of the Yama/CPP32 protease, caspase-3 (14). PARP's role in recognizing DNA breaks along with the fact that CPP32-mediated cleavage separates its DNA-binding domain from its catalytic domain, has led to the notion that PARP inactivation may be functionally important for efficient DNA fragmentation to occur during late phases of apoptosis (15). However, mice lacking PARP show no obvious defects in apoptosis (16), suggesting that it is not required for apoptosis to occur, although functional homologues could in principle be rescuing PARP.

A wealth of substrates continue to be identified as potentially important signaling intermediates. These include, among others, the nuclear lamins (17-19), the 70kD component of the U1 snRNP (20, 21), DNA-PK (22), Gas2 (23), D4-GDI (24), PKC-δ (25), SREBPs (26), Huntingtin (27), PAK2 (28) and RB (29,30). Biochemical isolation of other relevant substrates based on their activity to signal apoptosis using *in vitro* systems should provide a clearer picture of the nuclear events downstream of the cysteine proteases. A number of cell-free systems like chicken S/M extracts (13, 31), HeLa cell extracts (32), Xenopus extracts (33, 34), as well as extracts of other cells triggered to die in response to a variety of apoptotic triggers (35-37) have already been used to reconstitute portions of the apoptosis pathway *in vitro* and characterize novel apoptosis-inducing activities. One such activity was recently isolated from HeLa cell extracts and termed the DNA fragmentation factor or DFF (38). DFF is cleaved both *in vitro* (following addition of recombinant CPP32 to extracts) and *in vivo* (in U937 cells treated with Staurospaurine), and is required for chromatin condensation and DNA laddering to occur. Although the mechanism by which DFF induces DNA fragmentation remains unknown, this new protein is one of the first ICE-family protease substrate to date with a demonstrated role in signaling downstream events of apoptosis.

3.2 Channeling our attention on the Bcl-2 family

Bcl-2 is the mammalian homologue of *ced-9* which, in *C. elegans*, is required to protect cells that normally survive from undergoing programmed cell death (4, 39). It has been the focus of intense study ever since its demonstrated ability to rescue pre-B cells from apoptosis in response to IL-3 withdrawal (40). Originally identified as a result of the t(14;18) translocation in B-cell follicular lymphoma in which its juxtaposition with the IgH enhancer leads to dysregulated overexpression, Bcl-2's causal role in tumorigenesis was directly confirmed in studies where mice expressing a Bcl-2 transgene in lymphoid cells developed B-cell malignancies (41-43). Bcl-2 is now the prototype of a rapidly growing family of interacting proteins which share its ability to modulate apoptosis. A prevalent model has emerged by which a cell's threshold to apoptosis is determined by the levels of pro- and anti-apoptotic members which, through dimerization, act as a survival "rheostat switch" (44). The biochemistry through which Bcl-2 and its 'partners-in-crime' mediate their effects has only recently started to unfold.

Bcl-2 family proteins share several homology regions (Bcl-2-homology/BH domains) crucial for both their dimerization and apoptosis-modulatory functions (44). The BH domains along with the use of the yeast two-hybrid and other methods have been instrumental in the identification of novel interacting proteins such as Bax (45), Bcl-x (46), Bad (47), Bag-1 (48), Bak (49-51), Bik (52), Hrk/harakiri (53) and others. The pro-apoptotic Bax protein, cloned through its ability to co-immunoprecipitate with Bcl-2, was originally found to form homodimers as well as heterodimers with Bcl-2 via the BH1 and BH2 domains (45). Site-directed mutagenesis of these domains in Bcl-2 prevent heterodimer formation with Bax (or Bak) and abrogate its death-repressor activity (54). Interestingly, Bcl-2 mutations permissive for homodimerization, but not heterodimerization with Bax, also abolished Bcl-2 activity suggesting that Bcl-2 requires Bax to exert its death-repressor activity.

It remains unclear, however, who among the pro- and anti-apoptotic members are the key effectors in the apoptosis pathway. Indeed, unlike Bcl-2, not all members appear to require heterodimerization to carry out their protective effect. The Bcl-x transcript is alternatively spliced into a short and long form to yield both pro- and anti-apoptotic factors (Bcl-x_S & Bcl-x_L respectively) (46). While the long form resembles Bcl-2 in its dimerization preferences and anti-apoptotic capacity, Bcl-x_L mutants which prevent its interaction with Bax or Bak can still rescue Sindbis virus-induced apoptosis (55). The short splice form of Bcl-x, although lacking BH1 and BH2 dimerization regions, appears to mediate its death-inducing function by antagonizing Bcl-2 and Bcl-x_L (46).

A simple 'rheostat switch' model whereby both pro- and anti-death molecules require co-interaction to mediate their effects on apoptosis is not sufficient to explain the above observations. Alternatively, death-promoting and death-suppressing Bcl-2 family proteins may operate in mechanistically different ways. The phenotype of Bax knock-out mice which display both an excess and lack of cell death in different tissues supports this possibility (56).

A third region of Bcl-2 homology - BH3 - has been demonstrated to be essential for the activity of the pro-apoptotic proteins. Indeed, although all three BH1, 2, & 3 regions are involved in dimer interface as revealed by structural studies (57), expression of the BH3 domain of Bak alone is sufficient to induce apoptosis (58). A crystal structure of Bcl-x_L bound to a Bak peptide derived from the BH3 region demonstrates the importance of this domain in mediating protein-protein interaction (59). Bcl-x_L BH1, BH2, and BH3 domains form an elongated hydrophobic cleft as revealed by NMR and X-ray structures (57). When bound with Bcl-x_L, the BH3 region of Bak adopts an amphipathic alpha helix and mediates both hydrophobic and electrostatic interactions with the Bcl-x_L hydrophobic cleft. Mutations in residues observed to form specific contacts within this cleft abolish interaction of the two proteins (59).

Structure determination of Bcl-x_L, in addition to revealing the overall architecture of the BH dimerization domains, has also revealed a somewhat unexpected structural similarity of this protein to the membrane insertion domain of the diphtheria toxin and related colicins (60). Indeed, comparing Bcl-x_L (as well as other members like Bcl-2) with the diphtheria toxin reveals that both proteins have two central helices with apolar residues (alpha$_5$ and alpha$_6$ in Bcl-x_L) surrounded by three amphipathic helices (alpha$_1$, alpha$_3$, and , alpha$_4$ in Bcl-x_L)(57). The two central hydrophobic helices alpha$_5$ and alpha$_6$ of Bcl-x_L are long enough to span the length of the membrane, and, like the insertional domain of the diphtheria toxin, may require dimerization to form a pH-dependent membrane pore (61). By analogy with the functional role of the insertional domain in toxins, these observations raise the possibility that proteins of the Bcl-2 family may control the passage of ions or other components across the mitochondrial membrane. Alternatively, Bcl-2 family proteins may interact with and regulate the activity of other mitochondrial transport proteins via these helical domains. A recent report that Bcl-x_L forms pH-sensitive ion-conducting channels in synthetic lipid membranes has reinforced this hypothesis (62).

Until recently, Bcl-2's ability to regulate the apoptosis pathway remained a mechanistic enigma. Based on observations linking Bcl-2-mediated rescue of apoptosis with lower levels of oxygen free radicals as well as Bcl-2's localization to nuclear, ER and mitochondrial membranes, early studies advanced the possibility that Bcl-2 could function in an antioxidant pathway (63, 64). Bcl-2 and Bcl-x_L can prevent hypoxia-induced cell death, however, suggesting that they exert their anti-apoptotic activity by a mechanism other than modulation of oxygen free radicals (65, 66). Based on cellular fractionation studies as well as electron microscopic observation, others hypothesized that Bcl-2 may be involved in nuclear transport (67). The more recent discovery of the extended family of Bcl-2 dimerization partners led to a model whereby ratios of these molecules may determine a cell's susceptibility to apoptosis in response to a variety of apoptotic stimuli (44). While these observations suggest how regulation may occur within the family, they give no hint as to how these proteins are regulated by exterior signals as well as a possible connection with the central apoptosis machinery. In addition to their putative role as channel-forming assemblies, Bcl-2 family proteins have now been found to recruit and interact with several signaling proteins which may regulate their activities.

Although in many cases levels of pro-and anti-apoptotic Bcl-2 family proteins determine a cell's susceptibility to apoptosis, there exist several demonstrated instances where expression levels do not correlate with apoptosis propensity (68, 69). Post-translational modification has been put forth as a possible mechanism for modulating the death activities of these proteins. Recent evidence suggests that trophic factor-induced phosphorylation of both Bcl-2 and Bad may regulate their apoptotic functions. For both Bcl-2 and Bad, phosphorylation has been suggested to have an inhibitory effect. Bcl-2 phosphorylation on serine occurs in response to a variety of stimuli and often correlates with decreased anti-apoptotic potential (70, 71). Bcl-2 has also been proposed to recruit the Raf-1 kinase to outer mitochondrial membranes and induce phosphorylation of Bad (72). This phosphorylation triggers Bad's dissociation from Bcl-x_L and cytoplasmic association with the 14-3-3 protein (73). While the effect of these modifications on the apoptosis functions of Bcl-x_L or Bcl-2 are not yet fully understood, they may provide a link between growth factor pathways and regulation of the downstream apoptosis machinery.

An interaction between Bcl-2 family proteins, members of the ICE-family cysteine proteases, and the nematode protein CED-4 has recently been demonstrated by a number of groups (74-76). Considering the epistatic relationship of these genes in *C. elegans* whereby *ced-9* rescues cells from apoptosis by antagonizing the activity of *ced-3* and *ced-4* (4), a direct physical interaction between these proteins was tested. CED-9 appears capable of binding the death-inducing splice form of CED-4 (77) using the yeast two-hybrid system and *in vitro* binding studies (75, 76). Mutations in CED-9 which eliminate its anti-apoptotic activity were found to disrupt its ability to interact with CED-4. These studies point to the importance of CED-4 binding for CED-9 function, and raise the possibility that the CED-4 interaction domain of

CED-9 could potentially be used as a bait for identifying mammalian CED-4 homologues.

A key observation which allowed for a CED-4/Bcl-x_L/ICE interaction to be tested in a mammalian tissue culture system was that the nematode CED-4 protein is sufficient to induce apoptosis when overexpressed in mammalian cells (74). In addition, CED-4-induced apoptosis can be inhibited with Bcl-x_L or caspase inhibitors suggesting that in mammalian cells, CED-4, under control of the Bcl-2 family, induces death via ICE-family protease activation. Once this was established, Chinnaiyan *et al.* took advantage of mutant proteins with the binding but not the cytotoxic properties of wild-type molecules in order to overexpress and immunoprecipitate CED-4 without killing cells. They observed that wild-type CED-9 (or Bcl-x_L) coimmunoprecipitates with CED-4 when cotransfected into 293 cells. In agreement with the yeast two-hybrid studies, immunoprecipitation of CED-4 failed to bring down a CED-9 mutant (lacking BH1 and BH2 domains) which does not block CED-4-induced apoptosis. Similarly, the ability of Bcl-x_L mutants to inhibit apoptosis correlated with their capacity to interact with CED-4. Coexpression of pro-apoptotic Bcl-2-family members such as BAX, BAK or BIK disrupted the CED-4/Bcl-x_L interaction.

In addition to its ability to interact with certain anti-apoptotic Bcl-2 family proteins, CED-4 was also observed to immunoprecipitate with ICE family-proteases which contain a large pro-domain (such as ICE or FLICE). Although the functional significance of this binding as well as the mechanism through which CED-4/ICE interaction may activate the protease, these results place CED-4 as a central regulatory switch between the Bcl-2 family and the downstream executioner machinery. The importance of CED-4 as an adaptor between these two families is manifest in the fact that a CED-4 mutant which interacts with CED-3 (but not CED-9) reduces Bcl-x_L's ability to coimmunoprecipitate with ICE or FLICE. The fact that CED-4 has no known mammalian homologue explains why the link between these two central apoptosis families has remained elusive until now. The key was to test the activity of the nematode protein in the context of a mammalian cell culture system. Given its central role, CED-4 may present a new therapeutic target for intervention in cancer, for example, where some upstream signaling components of the apoptosis machinery are often disabled.

Finally, an additional signaling activity has recently been attributed to the Bcl-2 family, and involves the release from mitochondria of proteins which may be instrumental in downstream apoptosis events. Several studies have shown that cytochrome c is released from mitochondrial stores upon induction of apoptosis, and that Bcl-2 overexpression is able to prevent this release (78, 79). Interestingly, cytochrome c was previously implicated in signaling apoptosis through its ability, in combination with dATP, to trigger PARP cleavage in naive HeLa cell extracts (32). Research also indicates that Bcl-2 may exert its anti-apoptotic activity by blocking the release of other death signaling molecules from mitochondria such as an as-of-yet unidentified apoptogenic protease (80). Whatever normally causes the release of either cytochrome c or other signaling entities from mitochondrial stores upon induction of apoptosis, as well as the mechanisms through which these molecules activate the executioner remain to be discovered.

In conclusion, current data point in several directions for a functional role of the Bcl-2 family in apoptosis. Anti-apoptotic members of the bcl-2 family may exert their death-suppressing function through their ability to bind and sequester a CED-4 homologue in mammals. Binding of CED-4 by these proteins presumably interferes with its ability to bind and activate CED-3 homologues like ICE or FLICE. Through dimerization with Bcl-2 or Bcl-x_L, pro-apoptotic Bcl-2 proteins may, in turn, disrupt their ability to bind CED-4, thereby freeing it for interaction with executioner proteases. Such a model offers an attractive signaling pathway which provides a direct biochemical link between the Bcl-2 family of apoptosis modulators with the cysteine proteases, and fits well with the epistatic relationships described in nematodes. Nevertheless, while the interaction with CED-4, recruitment of Raf-1, formation of ion-channels, and ability to release cytochrome c (and perhaps other apoptosis effectors) from mitochondria are all reported activities of Bcl-2 family proteins, it remains unclear how these diverse activities converge to modulate apoptosis.

3.3 The Fas way to apoptosis

Cytokine-induced apoptosis by Fas or tumor necrosis factor (TNF) is now one of the best studied and understood biochemical pathways to cell death which directly links ligand-receptor interactions at the plasma membrane with components of the executioner machinery. During development of the immune system, over 90% of immature thymocytes are deleted as a result of unproductive T cell receptor rearrangements or because they become self-reactive as a result of this rearrangement. Selection can occur in the thymus, in the peripheral circulation, or following the immune response (in the case of mature T cells). Based on the phenotypes of *lpr* (lymphoproliferation) and *gld* (generalized lymphoproliferative disease) mice which harbor defects in the Fas and Fas ligand (FasL) genes respectively (81), Fas-induced apoptosis is thought to be involved in at least two of these processes: peripheral clonal deletion and downregulation following immune reaction (82, 83). In addition, the Fas pathway has been linked to several types of autoimmune disease such as ALPS (autoimmune lymphoproliferative syndrome) and autoimmune diabetes (84-87).

FasL is a member of the tumor necrosis factor (TNF) family of membrane and secreted proteins which is displayed on T cells following T cell receptor signaling (88-90). Fas (also referred to as APO-1 or CD95) is a receptor of the TNFR family of cell surface proteins (91, 92). While Fas and TNF receptors are abundantly expressed in a wide variety of tissues (thymus, liver, heart, kidney), FasL and TNF expression patterns (mostly in natural killer cells and activated lymphocytes) appear to be a more relevant indication of where Fas-mediated cell death occurs. Both FasL and TNF undergo metalloprotease-mediated cleavage from the membrane and therefore also exist as soluble forms. It remains unclear whether this cleavage extends their signaling range or whether the shedding of these molecules is a means of downregulating their activity (82). Until recently, the signaling mechanisms by which ligand/receptor interaction resulted in apoptosis remained unknown. The identification of the death domain dimerization motif in the cytoplasmic tails of Fas and TNFR has led to the rapid isolation of a number of crucial intracellular intermediates in the Fas pathway.

The death domain (DD) was originally recognized by homology between Fas and TNFR1. Mutagenesis studies of these receptors revealed this region of approximately 80 residues to be crucial for FasL/TNF-induced apoptosis (93, 94). Several groups identified proteins interacting with Fas and TNFR using the DD as a yeast two-hybrid bait: FADD/MORT1 (Fas-associated protein with death domain), TRADD (TNFR1-associated death domain protein), and RIP (receptor interacting protein) (95-99). The recent NMR structure of the DD reveals the biochemical basis for its ability to oligomerize and mediate signaling (100). In fact, the DD may be somewhat of a misnomer since it is found in a number of receptors not all of which appear to be involved in signaling apoptosis (101). In addition to this domain, the recognition of a death effector domain (DED) in FADD led to the isolation of the FLICE (FADD-like ICE)/MACH (MORT1-associated CED-3 homologue) protein (102, 103). Activation of Fas or TNFR1 is thought to result in the formation of a 'death-inducing signaling complex' (DISC) which leads to interaction between these proteins based on DD and DED interactions. In addition, the recently identified DR3/WSL-1/Apo-3 receptor homologous to TNFR1 and capable of inducing apoptosis via its interaction with TRADD/RIP raises the possibility that other receptors also mediate cytokine-induced cell death (104-106).

A current model for Fas-induced apoptosis involves trimerization of the receptor upon binding a FasL trimer followed by FADD/MORT1 recruitment to the receptor complex via death domain interaction. Binding of the FLICE/MACH protease to FADD/MORT1 via its death effector domain leads to its activation which, in turn, is likely followed by proteolytic activation of other ICE family members. The recent discovery of a new family of viral FLICE-inhibitory proteins (v-FLIPs) found in several γ-herpesvirus and the tumorigenic human molluscipoxvirus points to the importance of FLICE as a central target for inhibiting the Fas/TNFR1 apoptosis pathway (107). By interacting with FADD via their DED, these viral proteins prevent the FADD-dependent recruitment and activation of FLICE, and completely block apoptosis induced via Fas, TRAMP or the as-of-yet unidentified TRAIL receptor (108, 109).

In addition to its role in apoptosis, TNF also induces activation of the NF-kappaB transcription factor. TNFR1 signaling is not as clear and well understood as that of Fas. Following TNF receptor trimerization, both TRADD and FADD/MORT1 can be recruited. It is at this level that the pathways of apoptosis and NF-kappaB activation bifurcate, since TRADD has been shown to have both apoptosis and NF-kappaB signaling properties (97). TRADD interacts with the RIP serine/threonine kinase to induce apoptosis, as well as with members of the TRAF (TNF receptor-associated factor) family to mediate NF-kappaB activation (110). While RIP does not contain a DED, it is its DD which is required to induce apoptosis suggesting an interaction with another signaling molecule. Such a candidate molecule was recently identified as RAIDD (RIP-associated ICH-1/CED-3-homologous protein with a death domain), which in addition to interacting with RIP through its DD, contains a sequence homologous to the 'pro' domain of the ICH-1/CED-3 proteases (111). This region of homology mediates RAIDD's interaction with these proteases and likely leads to their activation. Therefore, at least two potential signaling links have been made between Fas/TNFR1 and the downstream executioner machinery: one via FADD/MORT1 and involving the FLICE/MACH protease, and the other via TRADD, RIP, and RAIDD to activate ICH-1.

Several important questions regarding the Fas/TNFR signaling remain unanswered. How is the decision between FADD/TRADD-mediated death and TRADD-mediated NF-kappaB activation made given that certain TRAFs (TRAF-2 for example) interact with RIP as well as TRADD (99)? Why have cells evolved ways of controlling these two pathways via the same receptor complex? Studies suggest that NF-kappaB activation likely results in the expression of a 'survival gene' since inactivation of this pathway (with IkappaB phosphorylation-defective mutants for example) leads to increased sensitivity to Fas and TNF (112, 113). The potential for modulating apoptosis in this fashion may extend to cancer therapy (114). The ability of NF-kappaB-induced gene expression to antagonize apoptosis induced by other triggers remains to be fully investigated.

Several studies have also demonstrated the requirement for ceramide in TNF-mediated cell killing, suggesting that the signaling pathways to the executioner may not be as direct as anticipated. Ceramide is a lipid second messenger derived from the sphingomyelin cycle, and has been implicated in mediating apoptosis in

response to a number of different triggers like radiation (115), chemotherapeutics (116, 117) as well as Fas and TNF (118, 119). Ceramide may nevertheless be a downstream signal in the Fas/TNF pathway since ICE-like activity appears to be necessary for ceramide generation in response to the Drosophila REAPER protein which, like Fas and TNFR, contains a death domain (101, 120). Similarly, an ICE-like activity has been suggested to be required for activation of the p38 stress activated kinase pathway by Fas (121).

Anther mystery is the mechanism through which FLICE becomes activated as well as its relevant substrates. Considering that other ICE-family proteases are typically activated following cleavage of their 'pro' domain (122), FLICE activation may similarly involve such a modification or cleavage reaction. The ICE protease cascade activated downstream of FLICE also remains poorly understood. The timing of CPP32-like and ICE-like protease activities following Fas ligation has been investigated using specific fluorescent substrates. These studies demonstrate that an ICE-like activity is transiently activated, whereas the CPP32-like activity accumulates during FasL/TNF-induced apoptosis (123). Nevertheless, it is likely that FLICE activation is still an early event in the pathway to irreversible death, given the ability of inhibitors such as Bcl-2 and Bcl-x_L to block Fas-induced death both *in vitro* and *in vivo* (124-126). In keeping with this notion and the recent emphasis on mitochondrial events in apoptosis signaling, cytochrome c function appears to be impaired in Jurkat cells undergoing Fas-induced death (127). Taken together these observations suggest that Fas/TNFR1-induced apoptosis signals may converge with other classical apoptosis pathways on a conserved executioner machinery.

3.4 The PI3-K/Akt survival pathway

During development of the nervous system, excess neurons are produced only to subsequently die by competing for a limited supply of secreted or membrane-bound neurotrophic substances. The 'neurotrophic hypothesis' was validated in the 1950s with the discovery of nerve-growth factor (NGF) - a polypeptide shown to be important for the survival of sensory and sympathetic neurons in vivo - and, more recently, with the identification of other neurotrophins such as BDNF, CT-1, NT-3 and CNTF (128). *In vitro* studies showed that PC12 pheochromocytoma cells required NGF for both differentiation and survival. While Ras/Raf/Erk was required for differentiation, PI3-K was found to be crucial for the survival effect of NGF. Indeed, the ability of NGF to prevent apoptosis could be blocked by PI3-K inhibitors wortmannin and LY294002 (129). Treatment of cells ectopically expressing the PDGF-R with PDGF induced PI3-K and protected against apoptosis while PDGF receptor mutants unable to activate PI3-K had no effect. With the recent discovery of the Akt kinase as a dowstream PI3-K substrate with anti-apoptotic activity, we are now one step closer to understanding how PI3-K is linked to the apoptosis machinery.

The protooncogene Akt (also referred to as protein kinase B) is a ubiquitously expressed serine/threonine kinase whose catalytic domain is homologous to that of PKA and PKC family kinases, and which contains an N-terminal Pleckstrin homology (PH) domain. Akt kinase activity is upregulated in the presence of serum in a variety of cell lines in a manner dependent on PI3-K. This was originally shown by Franke *et al.* using a PDGFR-negative cell line into which PDGFR mutants lacking PI3-K binding sites (Y740F & Y751F) were transfected. Alternatively, treatment of cells with the PI3-K inhibitor wortmannin also blocked Akt kinase activity (130). In addition, Akt activation was abrogated by point mutations in the PH domain, suggesting the importance of this domain in regulation of the kinase. These results along with those showing that PI3-K's ability to rescue cells from serum deprivation-induced apoptosis was independent of p70S6K (131) pointed to Akt as a likely candidate. Shortly thereafter it was discovered that IGF-1 or insulin-mediated survival of cerebellar cultures *in vitro* is dependent on Akt (132). Cells transfected with dominant-negative constructs of the kinase - PH domain alone or kinase-dead versions of the protein - induce apoptosis of neurons in the presence of growth factors. Conversely, neurons into which wt-Akt was overexpressed were resistant to serum-deprivation suggesting the role of this kinase in cell survival.

The ability of PI3-K and Akt to promote survival is not limited to cells of neuronal origin, however, as it was found that either activated PI3-K or Akt protects Myc-overexpressing Rat1 cells from undergoing apoptosis upon growth factor withdrawal. Indeed, as serum deprivation is not only an apoptotic trigger for neurons but also for transformation-selective apoptosis in fibroblasts (133), the ability of PI3-K/Akt pathway to rescue factor-dependent survival in these cells was tested by several groups (134, 135). Transfection of a constitutively active mutant of the p110 catalytic subunit of PI3K was found to block apoptosis induced by serum starvation in Myc-Rat-1 cells. V12 Ras's ability to rescue cells was also tested as the p110 point-mutant used in these experiments (p110 K227E) is thought to mimic the conformational change induced by binding of PI3-K to activated Ras. V12 Ras, however, enhanced rather than suppressed serum-deprivation induced death suggesting that Ras could mediate both pro-and anti-apoptotic signals. Using Ras mutants which differ in their ability to activate downstream effectors like Raf versus PI-3K, Kauffmann-Zeh *et al.* demonstrated that interaction with Raf promotes apoptosis while interaction with PI3-K is necessary for survival (135). In addition, Akt was necessary to mediate this effect as shown by the ability of dominant-negative (kinase-dead) Akt constructs to promote apoptosis.

These results have been confirmed in careful studies by Kennedy *et al.* demonstrating the ability of dominant active forms of Akt to protect cells from serum withdrawal-induced death (134). In the attempt to understand how Akt promotes survival, levels of Bcl-2 and Bcl-x_L proteins were tested and found to remain unchanged in Akt-overexpressing cells. Interestingly, however, while Bcl-2 can overcome apoptosis induced by Wortmannin, dominant negative (kinase-dead) Akt antagonizes the ability of Bcl-2 to rescue cells in low serum, suggesting an interplay between these proteins in preventing apoptosis. Perhaps not surprisingly, inhibition of PARP cleavage activity was shown to correlate with Akt-induced survival of these cells, establishing a role for Akt kinase upstream of apoptosis proteases with CPP32-like activity.

Additional pathways which could activate Akt in PI3-K-independent ways as well as Akt substrates important for preventing cell death remain to be discovered. So far, Akt is thought to be regulated via the action of at least two pathways - one involving PI3-K and the other dependent on an Akt-kinase (136). PI3-K regulates Akt by controlling phosphatidylinositol-3,4,5-P3 (PtdIns-3,4,5-P3) and phosphatidylinositol-3,4-P2 (PtdIns-3,4-P2) levels. Akt homo-oligomerizes upon binding PtdIns-3,4-P2 via its PH domain. This interaction, however, is not sufficient for full activation which requires additional phosphorylation in its catalytic loop (threonine 308) and on its C-terminal regulatory domain (serine 473). Since Akt is recruited to the plasma membrane through binding of its PH domain to PtdIns-3,4-P2, it may thus be brought into proximity of such a kinase. Although both MAPKAP-2 and the recently purified PDK1 kinase (activated by either PtdIns-3,4,5-P3 or PtdIns-3,4-P2) have been shown to phosphorylate Akt on its catalytic loop *in vitro*, it remains to be seen whether phosphorylation by these kinases plays a significant role *in vivo* (137). The role of the downstream GSK-3 in mediating Akt's apoptosis protective effects also remains unclear at the time. It will be interesting to see what links exist between the central apoptosis machinery and the Akt pathway, and whether Akt can protect cells from apoptosis induced by a variety of other triggers such as UV or ionizing radiation, although it has already been shown to be important in apoptosis upon loss of matrix adhesion (138).

4. APOPTOSIS AND CANCER

In the recent past, molecular oncologists focused their studies primarily on the cellular pathways controlling proliferation. Neoplastic disease was typically envisaged as resulting from defects in these pathways leading to excess cell division. By extension, cancer therapies, when successful, were thought to act by selectively targeting rapidly cycling cells. Therapeutic index could, for practical purposes, be conceptualized as a ratio of mitotic rates between normal and malignant cells. During the last five years, however, overwhelming evidence has accumulated which suggests that the other side of the balance - the rate of cell death - is just as important. A central theme in much of cancer research today is the ability of tumor cells to resist apoptosis in response to triggers which typically induce cell cycle arrest or death in their untransformed counterparts. We review the importance of apoptosis in modulating central processes in cancer biology such as tumorigenesis, anchorage-independent survival, tumor-induced angiogenesis, and antineoplastic therapies.

4.1 Apoptosis and tumorigenesis

Apoptosis is now widely accepted to play a role in tumorigenesis. In the same way that programmed cell death by apoptosis may have evolved as a mechanism for regulating cell numbers and their interactions during normal development, apoptosis may also serve to eliminate cells gone "awry" in the adult organism. In fact, many proteins which are typically known as regulators of cellular proliferation also are potent inducers of apoptosis. The *c-myc* protooncogene, an early growth response gene required for G1/S transition upon mitogenic stimulation, has been shown to play apparently divergent roles in modulating both proliferation and apoptosis.

Deregulated Myc expression is observed in a variety of tumors, and its enforced expression in animals leads to cellular transformation and tumorigenesis (139). In culture, fibroblasts transformed with Myc display increased growth kinetics when cultured in the presence of adequate serum growth factors. Upon serum withdrawal, however, they undergo rapid apoptosis, the kinetics of which correlate with cellular levels of Myc protein (133). In contrast, serum starvation in untransformed fibroblasts typically results in growth arrest. This transformation-selective response is also observed following a number of apoptotic triggers such as radiation and chemotherapy (see below). Myc activates apoptosis in other cell types as well (140, 141), and can cooperate with Bcl-2 to transform haematopoietic cells (142, 143). This scenario is observed *in vivo* during follicular lymphagenesis, whereby Myc overexpression leads to malignant growth instead of apoptosis when placed in the context of Bcl-2's anti-apoptotic effect (144).

While a number of other cellular as well as viral oncoproteins have dual roles in regulating both proliferation and apoptosis (145-147), many of these require p53 to promote cell death. The p53 tumor suppressor was recognized as a regulator of apoptosis following the observation that transfection or activation of wild-type p53 in tumor cells can result in rapid cell death (148, 149). Following these initial observations, a large number of studies demonstrated that most cytotoxic triggers like radiation or chemotherapeutics require functional p53 to induce apoptosis (150-153). Similarly, serum withdrawal-induced death in Myc-overexpressing cells or IL-3-dependent thymocytes is p53-dependent (154-156). Several studies in transgenic mice have confirmed the notion that p53's tumor suppressive role *in vivo* is closely linked to its ability to induce apoptosis (157, 158).

The adenovirus E1A gene, like Myc, sensitizes cells to apoptosis induced by serum withdrawal, ionizing radiation, and a variety of chemotherapy agents (159, 160). E1A is thought to mediate its effect on cell death at least in part by stabilizing p53 protein levels (160). Similar to the cooperation between Myc and Bcl-2 oncoproteins in follicular lymphoma, transformation of primary cells by adenovirus 5 requires both E1A and E1B (161). E1A's ability to stimulate proliferation through binding and inactivation of pRB is 'balanced' by its apoptosis-inducing activity in the absence of E1B. The E1B gene encodes two transforming proteins - 19K and 55K - both of which disable the p53 pathway to apoptosis (162). Therefore, in the absence of p53 mutations or the E1B proteins, cells overexpressing E1A eventually die by apoptosis (160). The human papilloma virus (HPV), responsible for the majority of cervical carcinomas, triggers an analogous molecular scenario upon infection (163). The E7 protein, like E1A, hijacks the cell cycle machinery through binding pRB, and will trigger apoptosis when expressed alone (164, 165). Coexpression of the E6 protein, which stimulates ubiquitin-mediated degradation of p53, cancels E7's cell death response and leads to cellular transformation (166).

Other proteins which directly regulate cell growth or growth response genes stimulate apoptosis when overexpressed, and point to a role for cell cycle regulation in the life/death decisions of a cell. Deregulated expression of proteins like pRB, cyclin D, or E2F-1 have been shown to affect apoptosis. In fact, characterization of E2F-1 knock-out mice by several groups reveals the paradox whereby this transcription factor can induce tumors as well as promote apoptosis and suppress proliferation *in vivo* (167, 168)! During the cell cycle the E2F-1 protein is released upon pRB phosphorylation in G1 to transactivate genes involved in S phase entry (169). When expressed alone in the absence of serum or in combination with a growth suppressor gene like p53, E2F-1 induces S phase entry followed by apoptosis (170, 171). Deregulated cyclin D1 expression has been shown to have the same consequences (172). As might be expected, however, the opposite effect on apoptosis has been observed when pRB is overexpressed in cells (173, 174). Therefore, in the context of a growth arrest signal like serum starvation, high p53 levels, or DNA damage (see below), deregulated expression of critical cell cycle progression effectors can trigger the cell into apoptosis. While these observations clearly indicate that signaling pathways to growth and apoptosis are tightly linked, they do not reveal the mechanism by which these proteins induce apoptosis. A key question therefore is whether genes like Myc, E2F, pRB and p53 mediate apoptosis through the same pathways by which they regulate the cell cycle.

Conflicting reports in the literature make it unclear as to whether apoptosis is triggered as a result of conflicting growth signals or whether the regulatory proteins we have discussed so far have distinct apoptosis-inducing activities of their own (175). Myc-induced apoptosis, for example, often correlates with elevated cyclin A expression and activation of cyclin A-dependent kinases cdk2 and cdc2 (176, 177). Inappropriate cell cycle activation of cdc2 leads to cell death (178), which, in other systems, can be suppressed by dominant negative cdks or cdk-inhibitors (179, 180). Similarly, overexpression of either cdc25 or ornithine decarboxylase - two of Myc's transcriptional targets - is sufficient to induce apoptosis (181, 182), and antisense inhibition of cdc25 can block Myc-induced cell death (147). While these studies indicate a role for these proteins in regulating apoptosis, other reports suggest that Myc-induced cyclin A and cell cycle progression does not mediate apoptosis (183, 184). The ability of Myc-overexpressing cells to be triggered into apoptosis throughout the cell cycle also supports this model (133, 185, 186). Studies on the transcription factor p53 and its mechanism of apoptosis-induction have also yielded contradictory results.

p53 regulates the expression of a number of genes in response to extracellular cues such as hypoxia or DNA damage (187). Two alternative cellular responses occur as a result of p53 induction: growth arrest in the G1 phase of the cell cycle or apoptosis. p53's transcriptional targets include Gadd45, a gene thought to be involved in DNA repair (188), $p21^{cip1/waf1}$, a G1-specific cdk-inhibitor (189), and Mdm-2, a negative feedback regulator of p53 (190, 191). While p53-mediated transactivation of these target genes is thought to be crucial for DNA damage-induced growth arrest (187), it remains unclear whether p53-mediated transcription of these genes is needed to signal apoptosis.

Myc-mediated apoptosis has been shown to require p53 in a manner independent of p21 induction (154), and p21-deficient mice - while defective in G1 arrest - undergo normal development and apoptosis (192). These data support the idea that p53's cell cycle regulatory function may be separate from its role in apoptosis. The *bax* gene was recently found to be a transcriptional target of p53 and is upregulated in response to a variety of p53-dependent apoptosis triggers (193). However, *bax* induction does not always correlate with p53-dependent apoptosis (145), and thymocytes from bax knock-out mice undergo normal p53-mediated apoptosis ((56). In some systems, p53-dependent apoptosis can occur without the apparent need of any new transcription or translation (154, 194). Some reports demonstrate that transactivation-defective p53 mutants lose their ability to mediate apoptosis induced by E1A (195), while others find that p53's transactivation activity does not correlate with apoptotic activity (196, 197). A human-tumor derived p53 mutant which retains cell-cycle arrest activity but is defective for apoptosis has also been described (198). p53-interacting proteins which may modulate its transactivation-independent activities have been identified and include WT1, the Wilms tumor suppressor gene

product (199), and the XPB and XPD helicases (197). Therefore, p53 may mediate both transactivation-dependent and independent signaling pathways to apoptosis.

In summary, many oncogenes which stimulate cellular proliferation also potently induce apoptosis. Although it still remains unclear whether these genes kill cells by mechanisms related to the manner in which they promote proliferation, tumors which exhibit deregulated expression of these oncogenes typically select genetic aberrations which inactivate apoptosis. p53 loss or high Bcl-2 levels are common examples of such aberrations, and often correlate with poor prognosis in the clinics (see below). These observations have provided insights on the *in vivo* process of 'multi-step' tumorigenesis, whereby loss of growth control as well as inactivation of the apoptosis pathway are central for the survival and proliferation of tumors.

4.2 Anoikis and anchorage-independence: implications for tumor metastasis

The extracellular matrix (ECM) mediates a number of pivotal processes from cellular migration during gastrulation to tissue homeostasis in the adult (200, 201). Only recently, however, has the ECM's importance as a suppressor of apoptosis been recognized (202). 'Anchorage-dependence' describes the requirement of certain cell types for ECM adhesion in order to proliferate. In the same way that trophic factors prevent apoptosis of "factor-dependent" cells, adhesion to the ECM - a process largely mediated by the integrin family of cell surface receptors (203) - has also been shown to be crucial for the survival of certain anchorage-dependent cells. The ability to grow imbedded within a semi-solid matrix has been extensively studied as a property cells can acquire during tumorigenesis, and changes in integrin expression patterns and levels during progression of tumors from benign to more malignant phenotypes have been described (204, 205). The realization that ECM adhesion is important for cell *survival* as well as proliferation has broadened our understanding of anchorage-dependence and motivated researchers to decipher the adhesion-mediated signaling pathways which inhibit apoptosis. Triggering adhesion-specific death pathways in transformation-selective ways may allow us to prevent anchorage-independent proliferation, a likely first step in the progression of tumors to metastasis.

Similar to the cellular responses to DNA damage or serum starvation, loss of matrix adhesion in primary cells can lead to one of two fates: growth arrest or apoptosis. Primary fibroblasts which are detached from ECM slow their rate of protein synthesis and eventually growth arrest in the G_1 phase of the cell cycle (206, 207). Indeed, integrin-mediated signals are known to regulate the cell cycle machinery at the level of the transcription and translation of various cyclins and cdk inhibitors (reviewed in 208). The behavior of primary fibroblasts contrasts with that of epithelial and endothelial cells which undergo rapid apoptosis when denied ECM adhesion - a process termed 'anoikis' (209).

Human umbilical vein endothelial cells plated on agarose or MDCK cells incubated in saturating amounts of either soluble ECM components or RGD inhibitory peptides are prevented from adhering and rapidly undergo anoikis (209-211). Several instances of developmentally-regulated programmed cell death triggered as a result of ECM degradation have also been reported. Mammary epithelial cells which require $beta_1$ integrin-mediated adhesion to survive are typically eliminated by apoptosis upon matrix degradation during mammary gland involution (212, 213). Similarly, during frog metamorphosis, the transcriptional activation of the matrix metalloproteinase stromelysin-3 by thyroid hormone results in apoptosis of primary intestinal epithelial cells due to remodeling of the ECM (214). During cavitation of the vertebrate embryo, inner endodermal cells which fail to contact the basement membrane are triggered into apoptosis, while those in direct contact with it are rescued (215). The mechanism by which integrin-mediated adhesion suppresses anoikis in these cell-types, however, remains unclear.

Given the central role of integrins in mediating adhesion to the ECM, their putative function in transducing survival signals has come under scrutiny. Transfection of certain integrins, for example, is sufficient to rescue melanoma cells from anoikis in a three-dimensional dermal collagen matrix (216). Focal adhesion kinase (FAK), which is known to bind and transmit signals from the integrins (217), has recently been linked to anoikis in primary cells (218, 219). The ECM has also been suggested to regulate the activity of common apoptosis signaling intermediates like p53 (220, 221), Bcl-2 (222), the Jun-N-terminal kinase (223), and ICE (212). Alternatively, integrins, like oncogenes, may signal through their effect on anchorage-dependent cell-cycle progression (208). It also remains to be seen which specific integrin heterodimer combinations mediate protective effects in distinct cell-types. Whatever the precise molecular signals may be, oncogenic transformation in epithelial and endothelial cells has been suggested as a mechanism for escaping this regulation, and achieving anchorage-independent survival and growth characteristics.

Oncogenic transformation of endothelial or epithelial cells as well as treatments which reduce cell-cell contacts between these cells rescues them from anoikis (202). Transfection of MDCK or human endothelial cells with v-Ha-*ras* or v-*src* oncogenes abrogates anoikis (209, 211). Similarly, inducible c-H-*ras* expression rescues rat intestinal epithelial cells cultured in three-dimensional growth conditions (224). Although the protective mechanism of transformation in these cell types is not yet fully understood, Ras-induced PI3-K leading to Akt

activation may be a crucial ECM-mediated survival signal (138). Ras has been suggested to harbor both pro- and anti-apoptotic potentials depending on the downstream cellular pathways it activates (135), and therefore differences in the effects of Ras-transformation on anoikis propensity may be more complex than anticipated. In certain contexts such as overexpression of nuclear oncogenes like Myc or E1A combined with serum starvation, Ras' pro-apoptotic signaling capacity may be 'dominant' over its ability to activate Akt. In fibroblasts which lose matrix adhesion, Ras-transformation in combination with either Myc or E1A does not rescue from anoikis, but instead profoundly activates it (221). Despite differences between cell types, matrix contact in primary cells may promote cell survival via integrin-mediated Ras activation of PI3-K/Akt (138).

Treatment of MDCK cells prior to matrix detachment with either scatter factor or the phorbol ester TPA - both of which lead to disassembly of epithelial intercellular junctions (225, 226) - also confers resistance to anoikis (209). Other developmental signals, in addition to scatter factor, which induce epithelial-mesenchymal transitions might similarly affect a cell's propensity to anoikis. Thus regulation of the survival signals from matrix attachment may themselves be subject to regulation during development. In contrast, a colon carcinoma cell line required intercellular contact for survival in suspension (227); integrins instead of intercellular junctions appeared to mediate this rescue. These observations suggest that a cell's response to the loss of ECM may depend on the status of cell-cell junctions and the signaling pathways they trigger. The loosening of intercellular contact is a likely prerequisite for migration as well as survival in suspension - two characteristics typically shared by primary fibroblasts. The effect of cell-cell contact on sensitivity to anoikis is likely to be cell-type specific. Unlike epithelial/endothelial cells which typically form tight cellular sheets *in vivo* as a result of intercellular connections, fibroblasts are highly motile and migratory and do not typically display extensive cell-cell contacts. Therefore, the apparent disparities in anoikis sensitivity observed between different cell-types in culture may reflect the *in vivo* behaviors and intracellular pathways of the cells.

In summary, detachment from the ECM triggers apoptosis in cell types such as epithelial and endothelial cells, while inducing cell cycle arrest in primary fibroblasts. Cell-type differences may also extend to transformed epithelial/endothelial cells, which may resist anoikis, and transformed fibroblasts, which are sensitized to it. The existence of ECM-mediated survival pathways suggests that tumor cells may acquire anchorage-independent growth and survival characteristics by circumventing an otherwise 'default' apoptotic pathway (228). This may be the case for the alpha$_5$beta$_1$ integrin, for example, which has been suggested to send growth arrest/apoptosis signals in fibroblasts or colon carcinoma cells when not ligated to fibronectin (221, 229). Interestingly, there is evidence to suggest that this same receptor induces apoptosis in hematopoietic cells when ligated to fibronectin (230). Therefore tumors arising from a variety of cell types may employ different strategies for circumventing anoikis: selection of mutations which result in constitutive activation of integrin-mediated survival pathways, or loss-of-function mutations in genes required to complete apoptosis in response to the loss of matrix adhesion. It will be interesting to see which signal transduction pathways underlie the basic behavioral difference between different cell types, and which genetic alterations are required to alleviate anoikis and allow for metastatic growth of tumors.

4.3 Hypoxia and angiogenesis

The influence of microenvironment on the carcinogenesis and progression of solid tumors is now well recognized. Transformed cells may not only accumulate genetic mutations which allow for unchecked cell cycle progression and anchorage-independent survival, but also undergo selective pressure to adapt to low nutrient/oxygen conditions. Hypoxia, which commonly occurs as a result of increasing tumor mass and lack of vasculature, is sensed by cells and leads to activation of the hypoxia inducible factor 1 (HIF-1) (231). This basic-helix-loop-helix transcription factor has been shown to have several transcriptional targets including the erythopoeitin (EPO) gene and vascular endothelial growth factor (VEGF) (232). The p53 tumor suppressor is also induced by hypoxia, although it remains unclear whether this occurs via HIF-1 (233).

Hypoxia is also a trigger for apoptosis (as well as necrosis) in transformed cells *in vitro* (234, 235), and correlates well spatially with areas of apoptosis *in vivo* (236). Likely as a result of its ability to modulate p53 protein levels and trigger apoptosis, hypoxia has been suggested to provide a selective pressure for expansion of apoptosis-resistant/p53-deficient cells at the core of solid tumors *in vivo* (236). Interestingly, solid tumors which have hypoxic regions typically carry a poor prognosis suggesting a possible correlation between hypoxia's ability to select for apoptosis-deficient cells and subsequent tumor resistance to chemotherapy or radiation.

In addition to conferring a survival advantage to p53-deficient tumor cells, hypoxia also induces a number of other cellular changes in order for cells to adapt to low nutrient/oxygen conditions (237). This adaptive response is carried out in a number of ways including upregulation of metabolic pathways (238, 239) as well as stimulation of angiogenic signaling pathways to increase tumor perfusion (240). The 'angiogenic switch' during tumorigenesis has been suggested to result from a shift in the local balance of angiogenic activators and inhibitors. Hypoxia has been shown to induce a number of genes whose expression may 'trip' the angiogenic switch (241). The VEGF promoter, for example, contains HIF-1 binding sites and is activated

by low oxygen as well as glucose starvation (242-244). Furthermore, oncogenic transformation by c-Ha-*ras* acts synergistically with the effects of hypoxia to stimulate VEGF reporter expression *in vitro* (232). Hypoxia's role in selecting for p53-deficient cells may also indirectly influence the local angiogenesis balance since mutation of p53 is typically associated with the loss of the angiogenic inhibitor thrombospondin-1 (245).

The effects of angiogenesis on the growth and metastatic potential of tumors have been extensively studied. Light microscopic and immunohistochemical studies have revealed a direct correlation between the invasive and metastatic potential of a tumor and the number and density of microvessels surrounding it (246, 247). The discovery of molecules such as angiostatin (248) and endostatin (249) have demonstrated how depletion of antiangiogenic factors following removal of a primary tumor can directly affect the angiogenic balance and stimulate the growth of local and distant metastases (250). Since preventing angiogenesis can block the growth of both the primary tumor and its metastatic outposts, it has been the focus of much attention in the development of new cancer therapies. Recent studies aiming to understand the mechanisms of angiogenesis and potential ways of interfering with tumor-induced vascularization have revealed that matrix adhesion of proliferating vascular endothelial cells is required for their survival (251).

Endothelial cell proliferation is stimulated by a number of soluble factors like the acidic or basic FGFs, VEGF, and other factors, most of which can be released by hypoxic tumor cells (240). Neovascularization, however, requires that endothelial cells accomplish a number of other tasks such as matrix degradation and migration to appropriate sites (252). These processes involve extensive changes in cell-matrix recognition pathways. For example, two cytokine-dependent angiogenic pathways in endothelial cells have been shown to induce expression of distinct integrin receptors. Basic FGF- or TNFalpha-induced angiogenesis was found to be dependent on the $alpha_V beta_3$ integrin, while VEGF- or TGFalpha-induced angiogenesis depended on $alpha_V beta_5$ (253).

$alpha_V beta_3$ and $alpha_V beta_5$ integrins may affect not only the migratory capabilities of cells but also their requirement for matrix in order to block apoptosis (251). This anchorage-dependent requirement appears to be specific to proliferating vascular endothelial cells implying that agents which target these integrin-ECM interactions would be quite specific and not affect normal vasculature. Short cyclic RGD-containing peptides which mimic the integrin's target recognition sequence on ECM proteins have been used in several mouse models and shown to be effective in blocking neovascularization (254, 255). Specifically, their mechanism of action in inhibiting angiogenesis has been linked to their ability to induce apoptosis of the newly forming vasculature. Therefore, the discovery of "anchorage-dependent" survival signals suggest new targets not only for blocking processes such as anchorage-independent tumor cell growth, but also for interfering with tumor-induced angiogenesis by initiating anoikis of proliferating vascular endothelial cells.

4.4 Antineoplastic Therapies

Accrued interest in deciphering apoptotic signaling pathways has, in part, been sparked by the recent understanding that successful antineoplastic therapies induce tumor cell apoptosis rather than killing them as a result of direct insult to DNA. Traditional radiation biology has hypothesized that ionizing radiation kills cancer cells by overwhelming their mitotic and/or metabolic needs, resulting in disarray and death. The same reasoning applied to chemotherapeutics which cause DNA damage or inhibit DNA modifying enzymes. These explanations, however, do not account for the fact that certain slow-growing tumors respond well to therapies while rapidly growing ones can be completely immune to the same treatments (175). Instead, examination of tumor samples using immunohistochemical assays specific for apoptotic cell death has revealed that death by apoptosis, not necrosis, often follows radiation or chemotherapy (256, 257). In addition, clinical evidence has suggested prognostic links between treatment outcome and distinct molecular genetic alterations which are known to disable the cellular apoptosis pathway (258). As noted above, the foremost example of such a mutated gene is the tumor suppressor p53.

Mutations in p53 are found in over 50% of all malignancies making it one of the most frequently aberrant genes in human cancer (259, 260). Although a number of different mutants of p53 are observed in human cancers, they cluster as missense mutations throughout the DNA binding region and in four 'hotspots' (261). Most of these mutations impair p53's ability to bind DNA and result in a loss of transactivation potential. Mice deficient in p53 show minimal developmental defects, but have a higher incidence of spontaneous tumors (262, 263), similar to humans with Li-Fraumeni syndrome (with p53 germline heterozygosity (264)). In addition to its undisputed role in tumorigenesis, p53 also has prognostic value in terms of response to therapy since most triggers of apoptosis induce and may require p53. From a clinical standpoint, mutations in p53 are usually a poor prognostic indication in a variety of tumor types including gastrointestinal, hematopoietic, breast, and genito-urinary cancers (152, 265-277). Conversely, highly curable tumors like pediatric ALL, testicular, and Wilms tumors often correlate with wild-type p53 status (175). In other tumors such as cervical cancer or sarcomas, although p53 status is wild-type, the protein may be sequestered in an inactive or degraded state due to the effects of viral oncoproteins such as HPV E6 and Mdm-2.

Cell culture studies using E1A/ras-transformed mouse embryo fibroblasts derived from either wild-type or p53 deficient mice have demonstrated that classical

therapeutics including etoposide and adriamycin as well as ionizing radiation employ p53 to induce apoptosis (150). These observations were subsequently confirmed *in vivo* in a nude mice solid tumor model (278). Similarly, transgenic mice carrying a p53-responsive lacZ construct confirm the *in vitro* data and demonstrate that p53 modulates radiation and drug sensitivity *in vivo* (279, 280). A number of genes in addition to p53 have been demonstrated to affect the cytotoxicity of drugs and radiation *in vitro*, and are suspected to have the same effects *in vivo*. Bcl-2 and Bcl-x_L, for example, inhibit chemotherapy-induced apoptosis in neuroblastoma cells (281, 282). The relevance of this observation is confirmed by the *in vivo* data that a significant number of primary neuroblastomas harbor elevated Bcl-2 levels which correlate with aggressive tumor behavior and poor prognosis (283, 284). Bcl-2 has been shown to modulate drug-induced apoptosis in other systems as well (285, 286) although, surprisingly, high Bcl-2 expression has been correlated with improved prognosis in breast cancer (287-291).

Since tumors accumulate mutations which increase their resistance to certain environmental apoptosis triggers like hypoxia as well as to classical antineoplastic therapies, it is crucial to learn how to manipulate the downstream apoptosis machinery in new, perhaps more direct, ways. Finding new therapeutic triggers which induce tumor cell apoptosis in a manner independent of p53 has important clinical implications and remains a central focus in this area of apoptosis research. Similarly, shifting the balance of Bcl-2 family proteins in tumor cells may also trigger cell death. Paclitaxel (also called taxol or taxotere), a drug which acts p53-independently in patients, may exert its cytotoxic effects through its ability to phosphorylate and inhibit Bcl-2 downstream of p53 (71). Interestingly, Bax has been shown to enhance the cytotoxicity of chemotherapeutics like paclitaxel, vincristine and doxorubicin (but not etoposide or hydroxyurea) in a p53-independent fashion (292). In addition, new drugs like Apoptin are being discovered which, at least *in vitro*, appear to kill transformed cells which lack p53 and overexpress Bcl-2 (293). Understanding how a tumor cell's environment makes it more or less susceptible to certain drugs may also add therapeutic strategies. For example, both hypoxia and the ECM have been shown to affect the cell's response to cytotoxic drugs or radiation.

How might ECM affect a cell's capacity to respond to classical antineoplastic therapies? An early study shows that intercellular contact can modify the cell's ability to repair DNA following radiation-induced damage (294). In some instances, blocking intercellular contact using integrin-targeted monoclonal antibodies is sufficient to induce apoptosis (227). Similarly, cells in contact with basement membrane ECM are more resistant to conventional therapies than ones lacking adhesion (295-298). The survival pathways activated upon ECM adhesion which have recently been the focus of anoikis research may therefore provide a broad resistance to cytotoxic treatments. Topotecan - a topoisomerase I inhibitor - is significantly more effective in inducing apoptosis in a variety of tumor lines when cells are treated in suspension (299). Therefore, therapeutic agents which specifically target tumor cell adhesion mechanisms similar to the cyclic RGD peptides used as angiogenesis inhibitors could potentially synergize with other apoptotic triggers. In combination with 'classical' therapies such as radiation or chemotherapeutics, these may potentiate their cytotoxic effects *in vivo* by downregulating survival signals like Akt (138) or Bcl-2 (222).

Hypoxia is another clear example of a microenvironement which directly determines the efficacy of certain treatments like radiation *in vivo*. Radiation is known to require oxygen in order to fix the DNA damage, a process referred to as the "Oxygen Effect" (300). Free radicals produced on the DNA can either decay or, in the presence of oxygen, form DNA-O_2 intermediates which typically result in double-strand breaks. Therefore, areas of low oxygen tension in solid tumors (caused by chronic or acute hypoxia) have been observed to be more resistant to the cytotoxic effects of radiation (301). Radiosensitizers - compounds which act as oxygen mimetics and potentiate the effects of radiation in such hypoxic environments - have been used in order to circumvent this problem (302, 303), and are under active clinical investigation.

Another approach whereby therapies may halt tumor cell proliferation and induce apoptosis has been to target the activity of dominant oncogenes. Farnesyltransferase inhibitors block the activity of the enzyme farnesyltransferase which modifies a number of small G proteins like Ras and trigger localization to the plasma membrane (304). Since this localization is required for Ras' signaling activities, inhibiting this process was hoped to abrogate its signaling potential and inhibit Ras-induced transformation. These inhibitors may also block Rho, another small G-protein involved in actin cytoskeletal organization (305). Other studies have confirmed a role for Rho in Ras-induced transformation (306) and shown that Rho can induce apoptosis via the production of ceramides (307). These studies indicate that other downstream effectors of Ras may also provide good therapeutic targets. The fact that fibroblasts forced into suspension appear more sensitive to farnesyltransferase inhibitors than adherent ones (308) again points to the importance of combining drugs with different targets in order to more potently induce apoptosis in a transformation-selective way.

5. CONCLUSIONS

Apoptosis research remains a field in full expansion with critical discoveries being reported almost weekly. However, despite the enormous progress which

has been made so far, we still know relatively little about the regulation and intersection of the central pro- and anti-apoptotic pathways inside the cell. Although recent biochemical data suggest ways in which Bcl-2 family proteins interact with and modulate cysteine protease activity, the precise biochemical mechanisms for this modulation as well as the role of putative signaling intermediates like cytochrome C remain to be discovered. Continued study of these cell death signaling pathways will hopefully provide a wealth of new targets for apoptosis-inducing therapies in the clinics. Finding cell death triggers which work independently of p53 may enhance the development of antineoplastic therapies. In addition, the study of p53 positive tumors which are resistant to apoptosis may assist in identifying additional death modulators downstream of p53. Understanding which oncogenically-induced apoptotic activities remain in tumor cells could further advance our ability to trigger apoptosis in a transformation-selective manner.

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