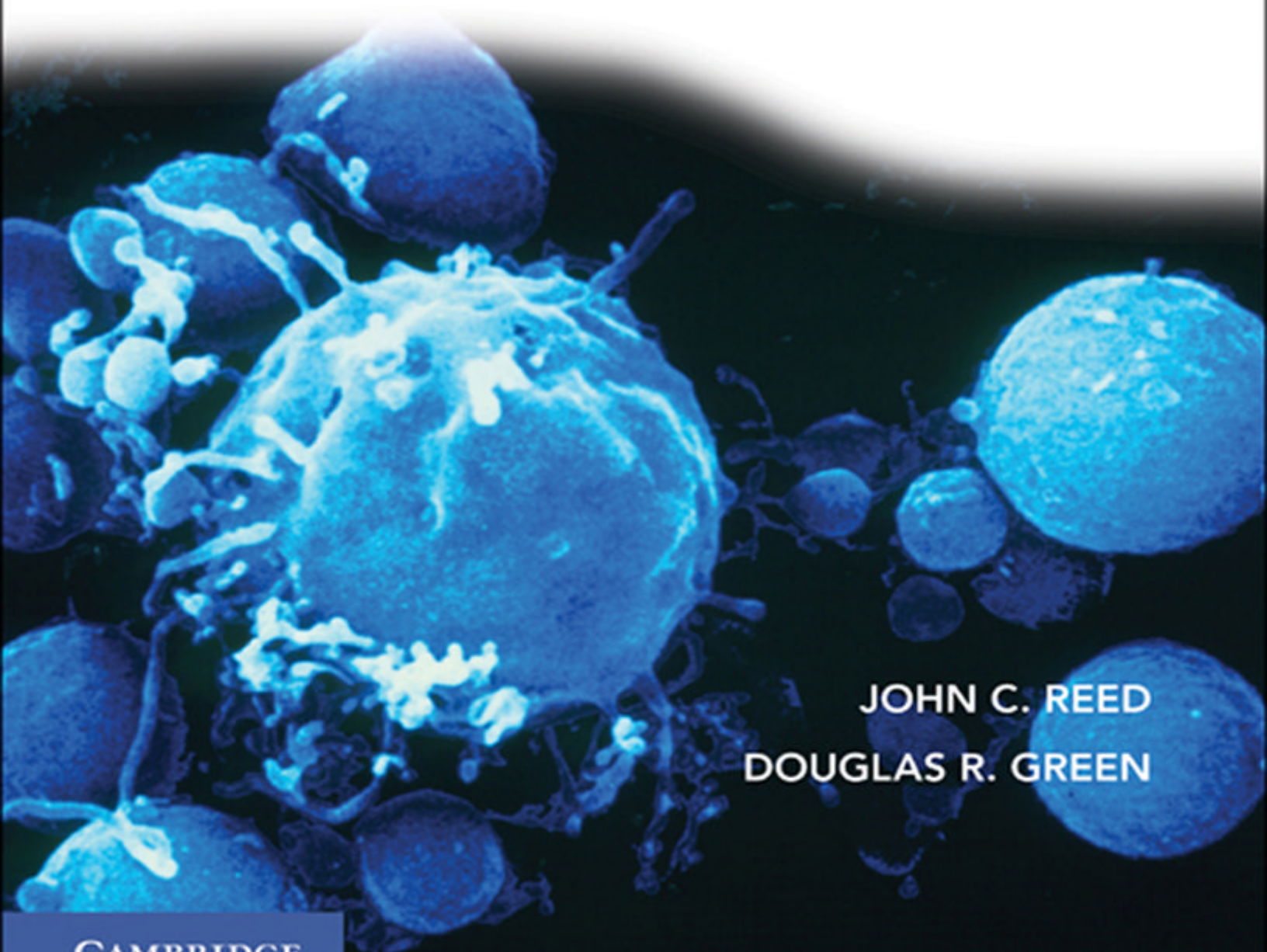


Apoptosis

Physiology and Pathology



JOHN C. REED
DOUGLAS R. GREEN

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APOPTOSIS

Apoptosis, or cell death, can be pathological, a sign of disease and damage, or physiologic, a process essential for normal health. This pathological dysregulation of cell death can be characterized by either too much loss of essential cells in the heart, brain, and other tissues with little regenerative capacity or too little cell turnover in self-renewing tissues, giving rise to cancer and other maladies. This is a process of fundamental importance for development and normal health, which is altered in many disease conditions. This book, with contributions from experts in the field, provides a timely compilation of reviews of mechanisms of apoptosis. The book is organized into three convenient sections. The first section explores the different processes of cell death and how they relate to each other. The second section focuses on organ-specific apoptosis-related diseases. The third section explores cell death in nonmammalian organisms that have served as popular models for research. This comprehensive text is a must-read for all researchers and scholars interested in apoptosis and cell death.

John C. Reed is Chief Executive Officer of the Sanford-Burnham Medical Research Institute. Dr. Reed is also Professor and Donald Bren Executive Chair at Sanford-Burnham, with adjunct professor appointments at several universities. Dr. Reed and his research team have contributed more than 800 research publications to the literature. Their work is among the most highly cited in all of science worldwide. Dr. Reed is the recipient of numerous awards and honors and has been awarded more than eighty research grants for his work. He is a named inventor for nearly 100 patents and the founder or cofounder of four biotechnology companies. Dr. Reed has served on the editorial boards of numerous journals; as an advisor to numerous public, private, and governmental organizations; and on the boards of directors of several public and private biotechnology companies and life-sciences organizations.

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APOPTOSIS

PHYSIOLOGY AND PATHOLOGY

Edited by

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1

Human Caspases – Apoptosis and Inflammation Signaling Proteases

Guy S. Salvesen

1. PROTEASE SIGNALING IN APOPTOSIS AND INFLAMMATION

In 1992 two groups independently reported the identity of a human protease responsible for activating the precursor of interleukin-1 β , naming it interleukin-1 β converting enzyme (ICE).^{1,2} Several months later, one of the key genes governing the commitment to apoptosis in *Caenorhabditis elegans* – *CED3* – was demonstrated to show homology with ICE.³ These publications initiated a successful search by many groups over the ensuing years for mammalian ICE homologs that should govern cell death. Today these proteases are known as caspases.⁴ Of the 11 caspases in humans, 7 are considered to be involved primarily in apoptosis, three are considered to be involved primarily in proinflammatory cytokine activation, and one is involved in keratinocyte differentiation (Figure 1-1). How cells learned to employ closely related proteases to execute two opposing phenotypes – apoptosis and inflammation – is strongly debated, and this chapter incorporates some discussion on this tricky issue.

Confirmation of the important roles of the caspases in the inflammatory cytokine response comes from gene ablation experiments in mice. Animals ablated in caspase-1 or -11 are deficient in cytokine processing,^{5,6} but without any overt apoptotic phenotype. The phenotypes of the apoptotic caspase knockouts are often gross and are sometimes antiapoptotic and vary from early embryonic lethality (caspase-8), to perinatal lethality (caspase-3 and -9),^{7,8,9} to relatively mild with defects in the process of normal oocyte ablation (caspase-2).¹⁰ Techniques in biochemistry and cell biology have allowed us to place the apoptotic caspases in two converging pathways (Figure 1-2). This core pathway probably represents a minimal apoptotic program, but

almost certainly the apparent simplicity is complicated by cell-specific additions that help to fine tune individual cell fates. Moreover, recent evidence clearly implicates caspase-8 in nonlethal roles in the control of cell proliferation.^{11,12}

1.1. Apoptosis and limited proteolysis

Apoptosis is a mechanism to regulate cell number and is vital throughout the life of all metazoan animals. Although several different types of biochemical events have been recognized as important in apoptosis, perhaps the most fundamental is the participation of the caspases.^{13,14,15,16,17}

The name *caspase* is a contraction of cysteine-dependent aspartate-specific protease⁴; thus their enzymatic properties are governed by a dominant specificity for protein substrates containing Asp and by the use of a Cys side chain for catalyzing peptide bond cleavage. The use of a Cys side chain as a nucleophile during peptide bond hydrolysis is common to several protease families. However, the primary specificity for Asp turns out to be very rare among proteases throughout the biotic kingdoms. Of all known mammalian proteases, only the caspase activator granzyme B, a serine protease, has the same primary specificity.^{18,19} Caspases cleave a number of cellular proteins,²⁰ and the process is one of limited proteolysis in which a small number of cuts, usually only one, are made. Sometimes cleavage results in activation of the protein, sometimes in inactivation,²¹ but never in degradation, because their substrate specificity distinguishes the caspases as among the most restricted of endopeptidases. This is an important distinction from the other well-known proteolytic signaling system – the proteasome – which permits signaling by wholesale destruction of regulatory proteins such as

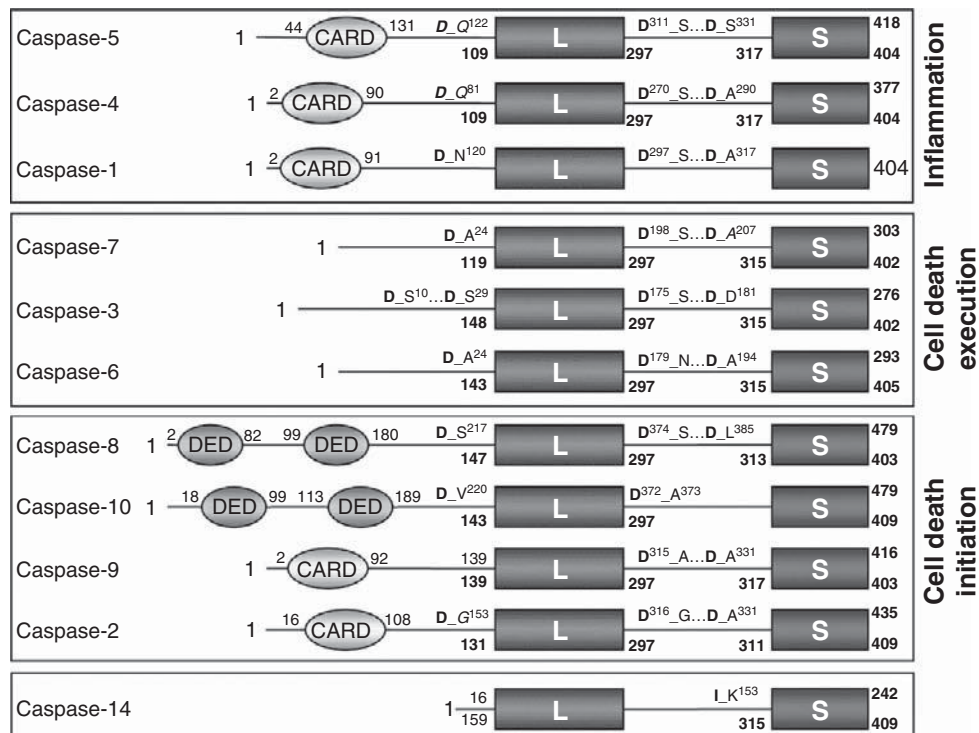


Figure 1-1. Domain organization of human caspases. Human caspases have been grouped according to their sequence similarities. Notice that sequence homology divides caspase-1 to -10 into three sub-families, in accord with the physiologic distinction between inflammatory, initiator, and effector caspases. In contrast to the widespread distribution of these family members, caspase-14 is mainly found in the epidermis, may be involved in keratinocyte differentiation, and is not activated *in vivo* at an Asp residue. The positions of suspected (*italics*) or known maturation cleavage sites are given, with the P₁ aspartate residue indicated by "D." Numberings correspond either to the Swiss-Prot entries, with the exception of caspase-10, for which the sequence of the more commonly expressed isoform 10/a is given, or to the caspase-1-based numbering convention. The ovals are the recruitment domains, and the catalytic domain is designated in bars for the large and small subunit. Reproduced with permission from Fuentes-Prior, P. and Salvesen, G. S. (2004). The protein structures that shape caspase activity, specificity, activation and inhibition. *Biochem J* 384, 201–32. © The Biochemical Society (<http://www.biochemj.org>).

IκB in nuclear factor kappa B (NFκB) signaling and PDS1 in anaphase promotion.²²

1.2. Caspase evolution

The first bona fide apoptotic caspase, Ced3, was identified in *C. elegans*, and it appears that this organism requires only one caspase to execute apoptosis.³ Initially it was thought that as the complexity of primitive cell death pathways developed, so apparently did the number of caspases. *Drosophila* species have 7 caspases,²³ and humans have 11. But this simple picture needs to be revised in light of the presence of multiple caspases in organisms far more primitive than *C. elegans*. It is likely that *C. elegans* and *Drosophila* may have lost genes that encode the more complex apoptotic network of more primitive animals. For example, the primitive sea anemone contains 10 caspases, whereas deuterostomes have seen an expansion to 42 members of the caspase

family.²⁴ Presumably, the multifaceted apoptotic response is more ancient than previously thought, because this expansion is not restricted to caspases but is also found in Bcl2 family members.

Mapping the inherent substrate specificity of caspases has allowed some broad consensus to be recognized.²⁵ These consensus also allow apoptotic caspases to be distinguished from proinflammatory caspases, because the latter have a rather distinct specificity that presumably allows them to carry out their job without threatening cell viability. Interestingly, there seems to have been a parallel evolution of apoptotic caspases along with their substrates. Consensus caspase targets in humans such as nuclear lamins and poly (ADP)ribose polymerase have easily recognizable caspase cleavage sites in *Drosophila*, but apparently not in organisms such as yeast and plants, which lack an apoptotic pathway. Indeed, although yeast and plants are known to employ programmed cell death, they do not contain

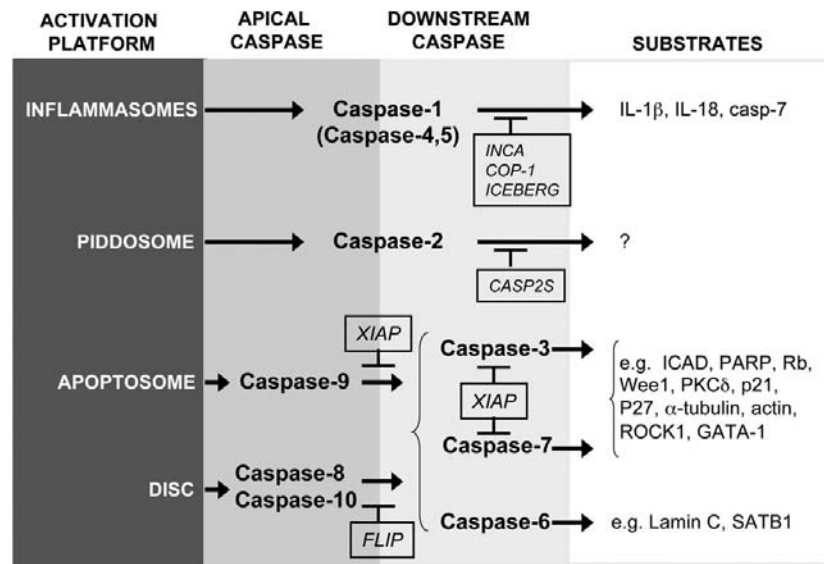


Figure 1-2. Activation pathways and substrates. An oligomeric protein platform activates an apical caspase, which then cleaves specific caspases. The apoptotic apical caspases require an intermediary step through the direct activation of downstream caspases, creating a two-step pathway that amplifies the apoptotic signal and allows for additional regulation points. The inflammatory caspases seem to use no intermediate, although the biochemical relationship of caspase-1, -4, and -5 is still obscure. A few caspase substrates are shown, but many are yet to be discovered. Caspase inhibitors, shown in boxes, regulate the activation pathways. Note that, although the inflammatory caspases are placed on a separate pathway, activated by recruitment to inflamasomes, they are able to activate caspase-7 during acute inflammation models in vitro, leading to the suggestion that the inflammatory network may feed into the apoptotic network in this manner.⁸⁶ Reproduced with permission from Pop, C. and Salvesen, G., Human caspases: activation, specificity, and regulation. *J Biol Chem* 284, 21777–21781. From the American Society for Biochemistry and Molecular Biology.

caspases, and the concept of apoptosis in these forms of life is probably wrong. They use other mechanisms to kill off their cells, such as the hypersensitive response in plants.^{26,27}

2. ACTIVATION MECHANISMS

Akin to the classic multistep proteolytic pathway of coagulation, downstream caspases are activated by proteolysis, but upstream ones, having no protease “above” them, must respond to an activating signal by another mechanism. Some time ago it was thought that all caspases were activated by proteolysis, but it has become clear that this is a minor mechanism in caspase activation, pertaining principally, at least in humans, to the three executioner caspase-3, -6, and -7. Structural information reveals that the conformations of zymogens are quite similar, as are the conformations of active forms. But the mechanisms that enforce the zymogen-to-active transition are substantially different between initiators and executioners.

2.1. Initiator caspases – activation by dimerization

In the latent state, initiator caspases are inert monomers that require homodimerization for activation. In vivo,

dimerization is facilitated by caspase recruitment to oligomeric activation platforms that assemble subsequent to an apoptotic signal. Adaptor molecules from the activation platform specifically bind caspase pro-domains such as the death effector domains (DEDs) in caspase-8 and -10 and caspase activation and recruitment domains (CARDs) in caspase-1, -2, and -9. The recruitment enforces a local increase in caspase concentration and generates activity by proximity-induced dimerization.²⁸ Each apical caspase has its own activation platform: the death-inducing signaling complex (DISC) recruits and activates caspases-8 and 10 and the apoptosome activates caspase-9 (Figure 1-2).

2.2. The activation platforms

The DISC formed by the Fas receptor, Fas-associated death domain protein (FADD), and caspase-8 is a highly oligomeric network of homotypic protein interactions that comprise the death domains of Fas and FADD. The crystal structure of the Fas/FADD complex shows a tetrameric arrangement of four FADD death domains bound to four Fas death domains. Fas appears to act as a mechanistic switch that prevents accidental DISC assembly yet allows for highly processive DISC

formation and clustering on ligation of the cognate death receptor.^{29,30}

Caspase-9 activation requires the cofactor Apaf-1, which, in the absence of an apoptotic stimulus, exists in a monomeric form.³¹ In the presence of cytochrome c, and either 2'-deoxy adenosine triphosphate (ATP) or ATP, the AAA+ ATPase domain of Apaf-1 oligomerizes to form a wheel-shaped signaling platform, the apoptosome.³² Each of these signaling platforms has the ability to recruit its cognate caspase through homotypic interactions of the N-terminal domain on the caspase.²⁸ Indeed, replacing the recruitment domain of caspase-9 onto caspase-8 allows caspase-8 to be activated at the apoptosome – giving support to the general induced proximity model for apical caspase activation by dimerization.³³

The PIDDosome may be involved in the activation of caspase-2, although in the latter case, scant structural evidence is available to substantiate this proposed mechanism. In some cases, specific adaptor proteins incorporated in the activation complex may direct the signaling toward different pathways. For example, under certain conditions, caspase-2 and caspase-8 can trigger either cell death or NF κ B survival pathway, although few mechanistic data have been put forward for the latter event.^{34,35}

The inflammatory caspases are probably activated by a similar induced dimerization mechanism. The multi-protein activation platforms are called inflammasomes, with affinity for the CARD pro-domains of caspase-1, -4, and -5.¹⁶ However, it is not clear whether the activation mechanism of inflammatory caspases occurs by enforced homodimerization or it is the result of heterodimerization with other components of the inflammasome, such that caspase-1 could heterodimerize with caspase-5, as has been seen for the caspase-8/FLIP heterocomplex, for example.³⁶ The structural and mechanistic aspects learned from work on various apoptosomes should prove interesting for a closely related family of proteins – the NOD-like receptors (NLRs). The NLRs encompass a glut of acronyms in the literature, including NOD (nucleotide-binding oligomerization domain), NALP (NLR-, LRR-, and PYD-containing proteins), NACHT (domain that is present in NAIP, CIITA, HET-E, and TP1), PAN (pyrin- and NACHT-domain family), and CATERPILLAR (CARD, transcription enhancer, R (purine)-binding, pyrin, lots of LRR) proteins. NLRs are key mediators in the innate immune system and are linked to inflammation, host defense, and a number of inflammatory diseases.^{37,38,39,40,41,42,43} All NLR proteins share a domain that is closely related to the NB-ARC AAA+ ATPase domain of Apaf-1.³⁸ Like Apaf-1, they are

thought to sense a signal – possibly a bacterial product – leading to their activation and the subsequent generation of activity in inflammatory caspases (caspase-1, -4, or -5) or kinases. These proteins can also be considered soluble receptor counterparts to the widely investigated Toll-like receptor transmembrane receptor family. Their domain architecture closely resembles the one seen in Apaf-1. Most NLR proteins possess leucine-rich repeats (LRRs) that are responsible for ligand binding in place of the WD40s of Apaf-1. It has been proposed that the NACHT domains, very much like Apaf-1, can form oligomeric inflammasomes to activate their target proteins via adaptor domains, such as CARD or pyrin domains.⁴⁴ Although detailed structural information about this process is not available yet, it is hypothesized that this family represents soluble receptors that use AAA+ related domains to assemble in a manner like that of Apaf-1 and thus activate their targets by oligomerization.⁴⁵

2.3. Executioner caspases – activation by cleavage

Short pro-domain (executioner) caspases occur as inactive dimers that require cleavage at a position within the catalytic domain to become active (Figure 1-1). The first step in activation, dimerization, has already occurred shortly after their synthesis, and the zymogens are restrained by a short linker that separates the large and small subunits of the catalytic domain. The clearest evidence for the activation of executioner caspases comes from the crystal structures of the zymogen and active forms of caspase-7,^{46,47,48} which reveal the molecular details of catalytic site formation on activation. Proteolytic processing of the linker allows rearrangement of mobile loops equivalent to the initiator caspases, favoring formation of the catalytic site.¹³ In vivo, the upstream activators of effector caspases are the apoptotic initiators (caspase-8, -9, -10) and the lymphocyte-specific serine protease granzyme B. Caspase-14, a short pro-domain caspase, requires both cleavage and dimerization for in vitro activation, although the natural activator has yet to be identified.^{15,49}

Although physiologic allosteric regulators of caspases are yet to be discovered, a cysteine protease from *Vibrio cholerae* that is distantly related to caspases uses a mechanism of allosteric activation induced by the host inositol hexaphosphate.⁵⁰ The intriguing possibility of caspase activation by an as yet undiscovered allosteric mechanism in vivo is suggested by the finding that the activity of caspase-1, -3, and -7 can be modulated in vitro by using ligands that bind next to the dimer interface, far away from the active site.⁵¹

2.4. Proteolytic maturation

Caspase activation is frequently followed by (auto) proteolytic cleavages called maturation events, often optional, chronologically distinct events, which are functionally distinct from activation. Most maturation involves trimming or removal of the pro-domain or cleavage of the inter-subunit linker. It is important to note that, in the absence of an activation process, maturation is unable to generate enzymatic activity. Caspases do not activate by pro-domain removal, an activation mechanism used by many other proteases.

As a source of new epitopes and arrangements, maturation has several important consequences at the cellular level. For instance, dimerization in the absence of maturation generates a form of active caspase-8 that can signal T-cell proliferation and activation, but not cell death. The apoptotic role of caspase-8 appears to require cleaved caspase-8 *in vivo*.⁵² Mechanistically, this auto-cleavage greatly stabilizes the caspases-8 catalytic domain, potentially enabling activity to linger in the cytosol once the protease is released from the DISC.⁵³ But it is not known whether simple stabilization by maturation could explain the contrasting functions of caspase-8 mentioned previously.

Maturation cleavage of the caspase-9 inter-subunit linker by caspase-3 lays the grounds for caspase-9 regulation by the endogenous inhibitor X-linked IAP (XIAP)⁵⁴ by exposing new epitopes necessary for XIAP binding. A clear role remains to be established for some maturation events, and it is entirely possible that most of these events are simply cleavage of innocent bystanders resulting from caspase activity. However, caspase maturation is a distinct process from activation, important for generating caspase stability or signaling downstream regulatory events.

3. CASPASE SUBSTRATES

The most essential feature of caspase substrate recognition is that caspases cleave after Asp residues. But other requirements need to be met to turn a peptide/protein into a good caspase substrate, both *in vitro* and *in vivo*. A peptide of sequence $P_4-P_3-P_2-P_1-P_1'$, with P_1-P_1' as scissile bond, is a caspase substrate when (1) the P_1 residue is Asp – with the notable exception of the *Drosophila* caspase Dronc, a caspase-9 relative, which cleaves *in vitro* just as well after Glu⁵⁵; (2) the P_1' residue is small and uncharged (Gly, Ser, Ala)⁵⁶; and (3) $P_4-P_3-P_2$ residues are complementary for interactions with the catalytic groove.²⁵ Optimal residues in P_4-P_2 turn a mediocre substrate (XXXD/G) into an excellent caspase substrate. For example, executioner caspases prefer DEVD/G peptides

to WEHD/G peptides, whereas the opposite is true for inflammatory caspases.²⁵ In the case of natural protein substrates, two more rules apply: (1) the substrate cleavage site (P_4-P_1') is exposed to the aqueous environment (this suggests that “loops” or “turns” of natural substrate fold are prone to be proteolyzed); and (2) caspases co-localize with their substrates.

At one time it was suggested that the sum total of proteolytic events of endogenous proteins by caspases defines apoptosis.^{57,58} An unexpectedly large number of proteins have been reported to be *in vivo* caspase substrates.^{21,59} Focused proteomics approaches reveal at least 400 cellular proteins that are cleaved in a caspase-specific manner after induction of apoptosis in cell culture.^{60,61,62} However, it is far from clear, and indeed rather unlikely, that all of these cleavages are required for apoptosis. Separating the cleavage events that cause apoptotic function/morphology from the collateral bystander events that are inevitable given the complexity of the human proteome turns out to be very difficult. Although the list of annotated caspase substrates continues to lengthen, most candidates lack functional evidence linking cleavage to a role in apoptosis. In principle, in-depth investigation of cleavage site mutants in cells and animals will help to gain a more realistic understanding of how caspases drive apoptotic cell death and which substrates are part of the apoptotic or inflammatory response.

An important aspect that needs to be kept in mind is that no specific artificial substrates/inhibitors for caspases exist. One can appreciate that IETD/G, theoretically preferred for caspase-8, could also be cleaved by caspase-3, as judged by the synthetic library data.^{25,56} However, extrapolating to data from real protein samples, a protein containing IETD/G should be a very good substrate for caspase-3, indistinguishable for caspase-8 activity. When the activity is measured in cell lysates, the high caspase-3 concentration masks the activity of other caspases, even if a so-called preferred artificial substrate is used.⁶³ Future attempts to divide caspase specificity in complex mixtures may follow the use of biotinylated probes that enable tagging of individual classes of proteases⁶⁴ or a combination of live-cell reporters and flow cytometry coupled with more selective caspase inhibitors.⁶⁵

4. REGULATION BY NATURAL INHIBITORS

The first level of regulating proteolytic pathways is by zymogen activation, but an equally important level is achieved by the use of specific inhibitors that can govern the activity of the active components. The

endogenous inhibitors of caspases, those present in mammalian cells, are members of the inhibitor of apoptosis (IAP) family. In addition to these endogenous regulators are the virally encoded cowpox virus CrmA and baculovirus p35 proteins that are produced early in infection to suppress caspase-mediated host responses. Each of the inhibitors has a characteristic specificity profile against human caspases, as determined *in vitro*, and these profiles, with few caveats,⁶⁶ agree with the biologic function of the inhibitors (reviewed in references 67, 68). Although IAPs and CrmA would be expected to regulate mammalian caspases *in vivo*, p35 would never be present normally in mammals, because it is expressed naturally by baculoviruses.

The best-characterized endogenous caspase inhibitor is the XIAP, a member of the IAP family. The IAPs are broadly distributed and, as their name indicates, the founding members are capable of selectively blocking apoptosis, having initially been identified in baculoviruses (reviewed in reference 69). Eight distinct IAPs have been identified in humans. XIAP (which is the human family paradigm) has been found by multiple research groups to be a potent but restricted inhibitor targeting caspase-3, -7, and -9 (reviewed in reference 70). Despite earlier claims, other members of the IAP family probably do not directly inhibit caspases (reviewed in reference 71) and have functions in addition to caspase inhibition because they have been found in organisms such as yeast, which neither contain caspases nor undergo apoptosis.^{72,73}

IAPs contain one, two, or three baculovirus IAP repeat (BIR) domains, which represent the defining characteristic of the family. The first BIR domain (BIR1) binds to TRAF2 and regulates interactions with tumor necrosis factor receptor complexes,^{74,75} the second BIR domain (BIR2) of XIAP specifically targets caspases 3 and 7 ($K_i \approx 0.1$ – 1 nM), and the third BIR domain (BIR3) specifically targets caspase 9 ($K_i \approx 10$ nM). This led to the general assumption that the BIR domain itself was important for caspase inhibition. Surprisingly, structures of BIR2 in complex with caspase-3 and -7 and BIR3 in complex with caspase-9 revealed the BIR domain to have almost no direct role in the inhibitory mechanism. Many of the important inhibitory contacts are made by the flexible region preceding the BIR domain.^{76,77,78,79} Interestingly, the mechanism of inhibition of caspase-9 by the BIR3 domain requires cleavage in the inter-subunit linker to generate the new sequence NH₂-ATPF.⁵⁴ In part this explains the cleavage of caspase 9 during apoptosis, which, as previously described, is not required for its activation. Paradoxically, it seems required for its inactivation by XIAP.

Significantly, neither CrmA-like nor p35-like inhibitors, which operate by mechanism-based inactivation,⁶⁷ have been chosen for endogenous caspase regulation; rather, IAPs have been adapted to regulate the executioner caspases. Although the reason for this is not certain, it seems likely that the IAP solution provides a degree of specificity that mechanism-based inhibitors cannot achieve. Thus XIAP inhibition of caspase-3 and -7 requires a nonstandard interaction with the extended 381 loop that is specific to these two caspases (reviewed in reference 67). Possibly, the 381 loop has evolved to achieve substrate specificity in the executioner caspases.⁸⁰ However, an equally likely possibility is that the 381 loop has been generated to enable the IAP scaffold to provide a unique control level over the execution phase of apoptosis. Adding to this level of sophistication, IAPs, but not CrmA nor p35-like proteins, are subject to negative regulation by IAP antagonists that go by the names of Hid, Grim, Reaper, and Sickie in *Drosophila* and Smac/Diablo and HtrA2/Omi in mammals (reviewed in references 69, 70).

Inhibition by decoy proteins uses proteins structurally related to caspase pro-domains, competing for the same adaptors within activation platforms. Thus, from a semantic viewpoint, they are not inhibitors, but rather “activation preventers.” FLICE inhibitory protein (FLIP), a pseudo-caspase-8 with a nonfunctional catalytic domain, precludes caspase-8 recruitment to the DISC. Similarly, caspase-1–related proteins such as COP1, INCA, or ICEBERG bind to the caspase-1 pro-domain via CARD–CARD interactions and prevent its recruitment to inflammasomes.⁸¹

The final mechanism of caspase regulation involves degradation via the proteasome, an eventual fate suffered by many cellular proteins. Activated caspases are ephemeral species inside the cell and have a more dynamic turnover than the inactive zymogens,⁸² and it has been suggested that the proteins responsible for their rapid removal are IAPs (mentioned previously). In addition to the defining BIR domain, many IAPs also contain really interesting new gene (RING) and ubiquitin-associated domains that are involved in ubiquitin ligation.^{83,84} Although it is somewhat controversial regarding whether these domains target the IAPs themselves, or cargoes such as caspases, the IAPs are currently the clearest candidates for removal of active caspases before they reach an apoptotic threshold. Consistent with this is the observation that mice with a deleted XIAP RING domain have elevated caspase activity in a subset of cells, implying a physiologic requirement of XIAP ubiquitin-ligase activity for caspase removal.⁸⁵

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2

Inhibitor of Apoptosis Proteins

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Inhibitor of apoptosis proteins (IAPs) constitute a family of apoptosis-suppressing proteins that contain at least one copy of a conserved domain called baculoviral IAP repeat (BIR), a zinc-binding fold involved in protein interactions. Humans and other mammals contain multiple genes encoding IAP family members, providing a diversity of variants with both common and specialized functions. IAPs are known for their ability to bind certain caspases, which are proteases responsible for apoptosis. Several IAPs contain RING (really interesting new gene) domains that bind ubiquitin-conjugating enzymes (UBC), whereas others possess UBC catalytic domains. These attributes endow many IAPs with E3 ligase activity, implicating them in the ubiquitinylation and proteasome-dependent degradation of a variety of cellular substrates. In addition, several IAP family members have multifaceted functions as platforms for coordinating signal transduction events associated with activation of particular protein kinases. Finally, some IAPs have dual functions as regulators of cell death and cell division. In this chapter, we provide an overview of IAP family proteins, including their structures and domain organizations, biochemical and cellular functions, intracellular locations, post-translational modifications, and relevance to disease.

1. THE BIR DOMAIN DEFINES MEMBERSHIP IN THE IAP FAMILY

The IAPs are structurally defined by their BIR domains and functionally defined by their ability to block apoptosis. The evolutionarily conserved BIR domains are located at the N-terminus of all IAP family members and are present as a single copy or in groups of two to three tandem repeats. BIR domains are composed of ~70 amino acids and contain the signature

sequence CX₂CX₁₆HX₆C. Each BIR domain folds as a three-stranded β -sheet with four to five α -helices, which pack tightly to form a hydrophobic core. The BIR structure is stabilized by a single zinc molecule coordinated by three cysteines and a histidine (Figure 2-1). BIR domains mediate protein-protein interactions among themselves and other proteins. However, not all BIR-containing proteins are apoptosis suppressors in all species in which they occur. The roles of some mammalian BIR-containing protein in apoptosis inhibition, for example, may be indirect or of questionable physiologic relevance, and BIR-containing proteins of some lower organisms (e.g., yeast, worms) most certainly are unrelated to control of apoptosis.

In humans, eight genes encoding BIR-containing proteins have been identified (Figure 2-2), and all of these have been reported to suppress apoptosis – at least when over-expressed in cultured cells. The human IAPs include Apollon (BRUCE; BIRC6), cellular IAP1 and IAP2 (c-IAP1/c-IAP2; BIRC2 and BIRC3), IAP-like protein 2 (ILP-2)/testis-specific IAP (Ts-IAP; BIRC8), Livin/melanoma IAP (ML-IAP; BIRC7), neuronal apoptosis inhibitory protein (NAIP; BIR1), Survivin (BIRC5), and X-linked IAP (XIAP; BIRC4). Orthologs of all eight of the human IAPs are found in mice; however, mice have an expanded NAIP locus that is highly polymorphic among mouse strains and that contains up to three copies of the gene, wherein several functional copies of NAIP are often expressed under different promoters.

Survivin (BIRC5) is the smallest of the human IAPs, containing a single BIR followed by a coiled-coil domain that contributes to dimerization of this protein. Livin (BIRC7) and ILP-2 (BIRC8) are slightly larger, containing a single BIR followed by a RING domain. The RING domain is characterized by the presence of six to seven cysteine residues and one to two histidines that



Figure 2-1. 3D Structure of XIAP BIR3. Ribbon depiction of the structural of BIR3 domain of XIAP (residues 255–346). The α -helices are shown in red, β -sheets are shown in green, zinc is shown in purple, and the side chains of the residues that chelate zinc are shown in yellow. Structure adapted from Sun et al. (2000) *J Biol Chem* 275:33777–81. © 2000 The American Society for Biochemistry and Molecular Biology. See Color Plate 1.

coordinate two zinc ions. This domain imparts E3 ubiquitin ligase activity on many proteins by virtue of its ability to bind UBCs. Apollon (BIRC6) also contains a single BIR domain but is a huge protein, containing a large C-terminal domain that contains a UBC catalytic domain. NAIP, c-IAP1, c-IAP2, and XIAP contain three

tandem copies of the BIR domain. In c-IAP1, c-IAP2, and XIAP, the BIR domains are followed by a ubiquitin-associated (UBA) domain and a RING domain. These proteins also have E3 ligase activity. In addition, c-IAP1 and c-IAP2 contain a caspase activation and recruitment domain (CARD) (Figure 2-2), presently of unknown function. Interestingly, however, many proteins involved in either apoptosis or innate immunity contain CARDs. The three BIR domains of NAIP are followed by a NACHT domain (homologous to the nucleotide-binding oligomerization domains of Nod-like receptor [NLR] family proteins), followed by several leucine-rich repeat (LRR) domains. In NLR family proteins, the LRRs bind pathogen-derived molecules, an event required for rendering NACHT domains capable of binding nucleotides and oligomerizing.

Marine organisms vary greatly in their BIR-encoding genes. The vertebrate fish species *Danio rerio* (zebrafish) contains four genes encoding BIRs, where the BIR is found associated with CARD, RING, and UBC domains, similar to land vertebrates. Similarly, the marine invertebrates *Ciona intestinalis* (ascidian) and *Strongylocentrotus purpuratus* (sea urchin) have at least three and two BIR-encoding genes, respectively. In these organisms, the BIR is found in association with CARD, RING, and UBC domains, similar to land animals.

The genome of the fruit fly, *Drosophila melanogaster*, contains four BIR-encoding genes with varying

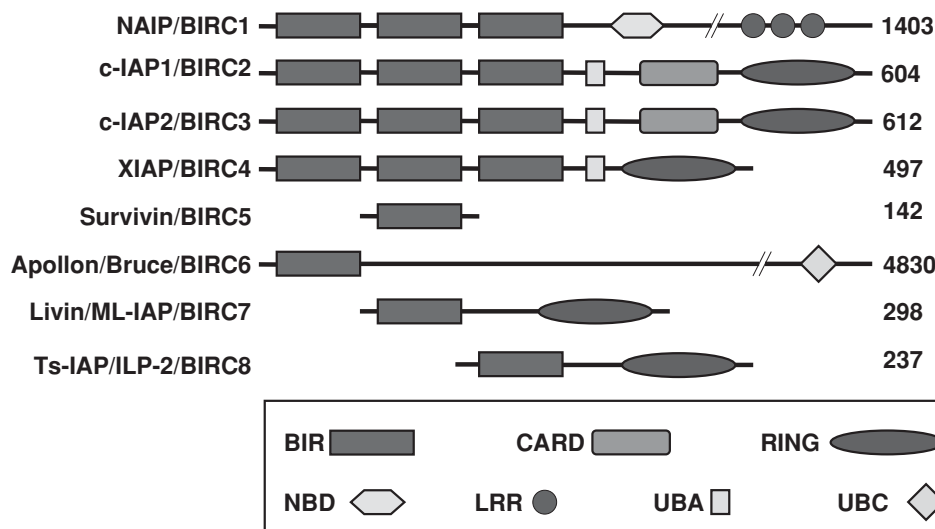


Figure 2-2. Domain organization of the human IAP family. The IAP family of proteins is structurally defined by their BIR domains. The human IAPs possess either one (survivin, Apollon, Livin, Ts-IAP) or three tandem BIR domains (NAIP, c-IAP1, c-IAP2, XIAP), indicated by red rectangles, CARD domains by green rectangles, RING domains by dark blue ovals, NBD domain by yellow hexagon, LRR domains by purple circles, UBA domains by teal squares, and UBC domains by light blue diamonds. (left) BIR, baculoviral IAP repeat; BIRC, baculoviral IAP repeat containing; c-IAP, cellular IAP; IAP, inhibitor of apoptosis; NAIP, neuronal apoptosis inhibitory protein; Ts-IAP, testis-specific IAP; XIAP, X-linked IAP. (right) The number of amino acids present in the respective human IAP family members is indicated. See Color Plate 2.

Table 2-1. Summary of human IAP family members and their various functions

IAP	Functions
NAIP	Innate immunity by detecting intracellular flagellin leading to caspase-1 activation
c-IAP1	Binds caspases-3, -7, -9 Sequesters SMAC Binds TRAF1/TRAF2 NF- κ B regulation Ubiquitinates substrates
c-IAP2	Binds caspases-3, -7, -9 Sequesters SMAC NF- κ B regulation Ubiquitinates substrates
XIAP	Inhibits caspases-3, -7, -9 Binds TAB/TAK NF- κ B regulation MAPK activation Copper homeostasis Ubiquitinates substrates
Survivin	Mitosis and cytokinesis regulation Binds XIAP, c-IAP1, c-IAP2
Apollon	Binds caspase-9 Ubiquitin conjugation Cytokinesis
Livin/ML-IAP	Binds caspase-9 Ubiquitinates substrates
Ts-IAP/ILP-2	Binds caspase-9 Ubiquitinates substrates

functions: *DIAP1* (*Drosophila* IAP-1), *DIAP2*, *deterin*, and *dBruce*. *DIAP1* (*Drosophila* IAP1) contains two BIR domains and a C-terminal RING domain. *DIAP2* contains three BIRs and a RING domain. *Deterin* is a small, Survivin-like IAP. Finally, *dBruce* (the *Drosophila* ortholog of Apollon/BRUCE), contains a single BIR domain as well as the UBC domain, similar to its counterparts in mammals. The nematode *Caenorhabditis elegans* contains two gene-encoding BIR proteins: BIR-1 and BIR-2. The yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* each produce a Bir1p protein with two tandem BIR domains. Bir1p acts similarly to mammalian Survivin in that it helps regulate the cell cycle (Figure 2-3).

2. CELLULAR FUNCTIONS AND PHENOTYPES OF IAPs

The cellular functions of IAPs include regulation of apoptosis but extend beyond cell death control – presumably reflecting the dual role that some of these proteins play in a variety of cellular processes. Table 2-1 provides a summary of the varying functions of the eight human IAP family members. All human IAPs are capable of

reducing apoptosis when over-expressed by gene transfection in cultured cells. Gene silencing by antisense oligodeoxynucleotides (AS-ODNs) or small interfering RNAs (siRNAs) has demonstrated a requirement for the IAP family members XIAP, c-IAP1, c-IAP2, ML-IAP, Livin, Apollon, and Survivin either for survival in culture or for resistance to certain apoptotic stimuli among various tumor cell lines. Over-expression of IAPs blocks apoptosis induced via the extrinsic pathway (tumor necrosis factor [TNF] family death receptors), intrinsic pathway (mitochondria-initiated), or both, depending on the specific IAP and the cellular context. For example, XIAP suppresses both extrinsic and intrinsic pathways, whereas Survivin, Livin, and ML-IAP have been reported to preferentially or exclusively inhibit the intrinsic pathway.

Certain BIR domain-containing proteins regulate cell division. In this regard, Survivin plays a role in chromosome segregation and cytokinesis, displaying a pattern of expression different from other IAPs in that it is expressed at high levels in embryonic tissues and in transformed cells. Survivin is not expressed in normal interphase cells of mammals, but its expression increases markedly during G2-M phase of the cell cycle in dividing cells. Survivin is required for proper chromosome segregation at the metaphase to anaphase

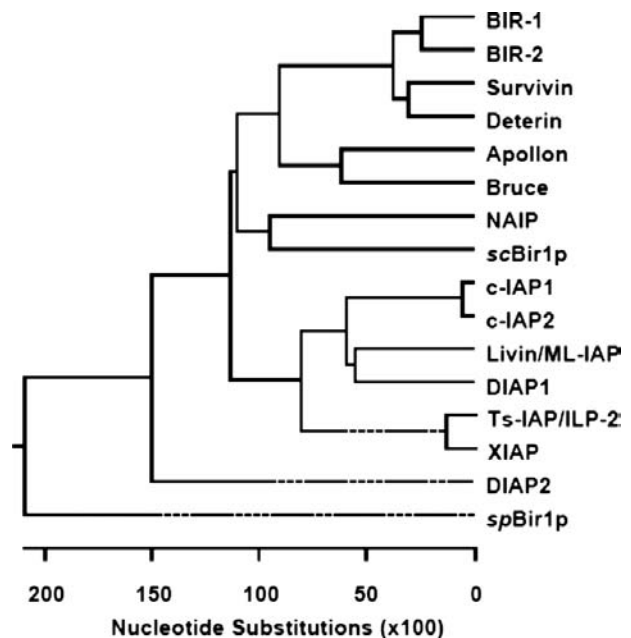


Figure 2-3. Comparison of BIR domains. Phylogenetic relationship of the IAP family of proteins is presented based on the sequences displayed in the MegAlign (DNASTAR) document using the CLUSTAL method. Full-length human BIR domain containing proteins: Livin, c-IAP1, c-IAP2, Ts-IAP, XIAP, Apollon, survivin, and NAIP. *Drosophila melanogaster* proteins: DIAP1, DIAP2, deterin, and BRUCE. *Caenorhabditis elegans* proteins: BIR-1 and BIR2. Yeast proteins: *Schizosaccharomyces pombe* (*sp*)Bir1p and *Saccharomyces cerevisiae* (*sc*)Bir1p.

transition and is essential for cytokinesis during telophase, when replicated daughter cells split. Similar roles have been reported for the Survivin ortholog of flies (deterin) and the BIR family proteins of worms (*C. elegans*) and yeast (*S. cerevisiae*; *S. pombe*) with respect to both mitosis and meiosis, suggesting an ancient role for these BIR-containing proteins in cell division. For example, *S. cerevisiae* Bir1p null mutant strains display instability of the yeast mini-chromosome, a chromosome mis-segregation phenotype.

Several IAPs play roles in signal transduction, as described in Section 7 in more detail. The signaling pathways affected by IAPs include nuclear factor kappa B (NF- κ B) and stress kinases (c-Jun N-terminal kinase [JNK]; p38 mitogen-activated protein kinase [MAPK]). XIAP has been identified as a critical component of signaling by certain bone morphogenic protein (BMP) receptors, such as BMP type I. The c-IAP1 and c-IAP2 proteins have been implicated in signal transduction by certain TNF family receptors.

NAIP is unique among mammalian IAPs in that it is both a BIR-containing IAP and also a member of the NLR family of innate immunity receptors (containing NACHT and LRR domain), which function in host–pathogen responses. The LRRs of NAIP are thought to operate as an intracellular sensor (receptor) of pathogens, in particular, bacterial flagellin. Evidence has shown that NAIP becomes activated on exposure to flagellin, resulting in activation of caspase-1 – a protease that cleaves and activates proinflammatory cytokines, pro-interleukin-1 β (pro-IL-1 β), pro-IL-18, and pro-IL-33. In *Drosophila*, the IAP family member DIAP2 is required for expression of endogenous antimicrobial peptides (AMPs), highlighting the role of this protein in innate immune responses. DIAP2 null flies infected with Gram-negative bacteria fail to mount an immune response and die. Recently, XIAP, cIAP1, and cIAP2 were also implicated in innate immunity by virtue of their role in signaling by NLR family members NLRC1 (NOD1) and NLRC2 (NOD2) [see section 7 for more detail]. Thus, connections between IAPs and innate immunity are robust – a feature often found in apoptosis-regulating proteins.

Proper copper homeostasis is essential to avoid toxic effects of excessive copper levels. Proteins that facilitate this balance work to export excess copper from cells, such as copper metabolism gene MURR1 domain containing 1 (COMMD1). XIAP is a rate-limiting component in determining intracellular copper concentration. XIAP directly binds copper and COMMD1 to mediate the ubiquitinylation and proteasomal degradation of COMMD1. Copper binding results in an apparent conformational change in XIAP, rendering it unstable and susceptible to proteasomal degradation.

3. IN VIVO FUNCTIONS OF IAP FAMILY PROTEINS

IAP family genes have been ablated in mice for six of the eight mammalian family members. Organism-wide gene ablation is embryonic lethal for Survivin (before E4.5) and BRUCE (E14.5 to the perinatal stage). Knock-outs of NAIP, c-IAP1, c-IAP2, and XIAP, in contrast, show no overt phenotype, but detailed examination reveals specific attributes. Mice with complete ablation of NAIP exhibit normal development yet show increased susceptibility to seizure-induced cell death. XIAP knockout mice display delayed lobuloalveolar development in the mammary gland yet no altered apoptotic sensitivity. Overall, however, mouse gene knockout studies for IAP family members NAIP, c-IAP1, c-IAP2, and XIAP have failed to produce blatant cell death phenotypes, suggesting perhaps that redundancy among IAP family members ensures cell survival during normal development and adult tissue homeostasis.

In addition to knockout mice, a variety of IAPs have been over-expressed in transgenic mice. Transgenic mice over-expressing c-IAP2 in the heart show resistance to apoptosis and cardiac dysfunction after ischemia/reperfusion injury. Using a cochlea-specific promoter to over-express XIAP in the inner ear, hearing and hair cell loss in the cochlea were reduced when compared with control mice. Additionally, high levels of human XIAP mRNA expression are present in developing T cells of the thymus and peripheral lymph nodes, and transgenic mice over-expressing an XIAP transgene (under the control of a T-cell-specific promoter) exhibit accumulation of thymocytes and/or T cells in primary (thymus) and secondary (spleen) lymphoid tissues, providing evidence that XIAP plays a role in the homeostatic balance of lymphocyte populations.

Although complete ablation of *Survivin* is embryonic lethal, a number of conditional knockout models exist, emphasizing the important role of this protein in normal physiologic development. Early deletion of *Survivin* in thymocytes of mice shows pre-T-cell receptor proliferation checkpoint arrest, whereas its loss at later stages results in normal thymic development, with neither leading to an increase in apoptotic sensitivity. In neuronal precursor cells, conditional deletion of *Survivin* (at day E10.5) leads to massive apoptosis, and affected neonates die shortly after birth as a result of respiratory insufficiency.

In zebrafish, knockdown of *Survivin* using AS-ODN (based on morpholino chemistry) in embryos results in reduced eye and head size and also causes defective angiogenesis. In addition, zebrafish with null mutations in *c-IAP1* display abnormal vascular development, further suggesting a role for IAPs in angiogenesis

and blood vessel development, maturation, or maintenance.

Knockout studies show that DIAP1 is required for the survival of many cell types in the fly. During larval development, knockout of DIAP1 in the *Drosophila* S2 cell line or a DIAP1 null mutation results in widespread caspase-dependent cell death in the absence of any extrinsic cell death signals. Interestingly, DIAP2 knockout flies exhibit no gross cell death-related phenotypes.

In *C. elegans*, inhibition of BIR-1 expression using RNA interference (RNAi) does not affect apoptosis in adult somatic or germ cells. However, in the embryo, the lack of BIR-1 expression results in early lethality and a failure to complete cytokinesis.

4. SUBCELLULAR LOCATIONS OF IAPs

Clues to the functions of some IAPs can be found in their subcellular locations. Among the most striking is Survivin, which localizes to mitotic structures in dividing cells. Survivin is associated with the kinetochores of metaphase chromosomes and is recognized as a chromosomal passenger protein. As cells divide, Survivin leaves the chromosomes, moves to the microtubules during anaphase, and localizes to the midbody microtubules during telophase, where it concentrates until cytokinesis is completed. When Survivin production is perturbed, other components of the chromosomal passenger complex (CPC), such as inner centromere protein (INCENP), Aurora kinases, and Borealin, fail to localize properly to the centrosomes (kinetochore), resulting in chromosome segregation defects and sometimes, depending on the cellular background, in cell death. Cells reaching telophase with deficient Survivin fail to complete cytokinesis and become either binucleated (or multinucleated with repeated attempts at division) or tetraploid (and eventually aneuploid with successive attempts at cell division). Interestingly, c-IAP1 also localizes to midbody microtubules during telophase and reportedly associates with Survivin. Cells stably overexpressing c-IAP1 accumulate in G2-M phase, exhibit cytokinesis defects, and display a mitotic checkpoint abnormality, leading to polyploid cells when exposed to microtubule-targeting drugs. The fly Apollon/BRUCE ortholog, dBruce, also localizes to the midbody microtubule ring during cytokinesis, binding mitotic regulators and components of the vesicle-targeting machinery. Thus several IAPs appear to regulate events associated with cytokinesis, although specific details of mechanisms are lacking.

Several IAPs traffic between the cytosol and nucleus, including XIAP, c-IAP1, and c-IAP2. Translocation of XIAP from the cytosol to the nucleus has been associated

with induction of cell death. For example, during neuronal cell death as a result of hypoxia-ischemia, XIAP is reported to move into the nucleus in complex with an endogenous inhibitory factor, XAF1, which binds XIAP, stimulating its nuclear translocation. In contrast, a pool of c-IAP1 redistributes into the cytosolic compartment in a caspase-dependent manner after apoptotic stimuli activate extrinsic (TNF and TNF-related apoptosis-inducing ligand [TRAIL]) and intrinsic (ultraviolet irradiation and staurosporine) pathways.

Association of IAPs with organelles has also been reported. In cancer cells (but not normal cells), a pool of Survivin is localized to the mitochondria. Evidence from cell imaging, subcellular fractionation, and electron microscopy suggests that the pool of mitochondrial Survivin translocates into the cytosol in response to apoptotic stimuli, where it binds XIAP and other proteins to aid in suppression of apoptosis.

5. IAPs AS CASPASE INHIBITORS

IAPs are among the few types of cellular proteins that are capable of binding active caspases. In humans, XIAP is recognized as a potent inhibitor of effector caspases-3 and -7, as well as initiator caspase-9. Thus XIAP operates both within the intrinsic pathway, downstream of Apaf-1/cytochrome c to suppress apoptosis, and at the point of convergence of several apoptosis pathways, where caspases-3 and -7 operate as executioners of the cell death program. Dissection of XIAP has revealed that its second BIR domain (BIR2) and a short upstream sequence ("linker") N-terminal to BIR2 are necessary and sufficient for potent (low nanomolar and even sub-nanomolar) inhibition of active caspases-3 and -7. In contrast, the third BIR domain (BIR3) of XIAP is necessary and sufficient for potent inhibition of active caspase-9. BIR domains from c-IAP1, c-IAP2, Livin, Apollon, and ML-IAP have also been shown to bind specific caspases, although with lower affinity (micromolar). Structural studies have demonstrated that the higher affinity interaction of XIAP is caused by having two points of contact as compared with only one in the other IAPs. In this regard, all caspase-binding BIR domains contain a surface crevice that accommodates a tetrapeptide sequence corresponding to the N-terminus of the cleaved caspase's small subunit of the catalytic domain (Figure 2-4). The tetrapeptide sequence has been dubbed the IAP-binding motif (IBM). The IBM mode of binding is shared by all caspases that bind BIRs. XIAP, however, has two additional modes of binding. The linker associated with BIR2 binds across the active site of caspases-3 and -7, whereas an α -helix of BIR3 makes an additional contact with caspase-9. The

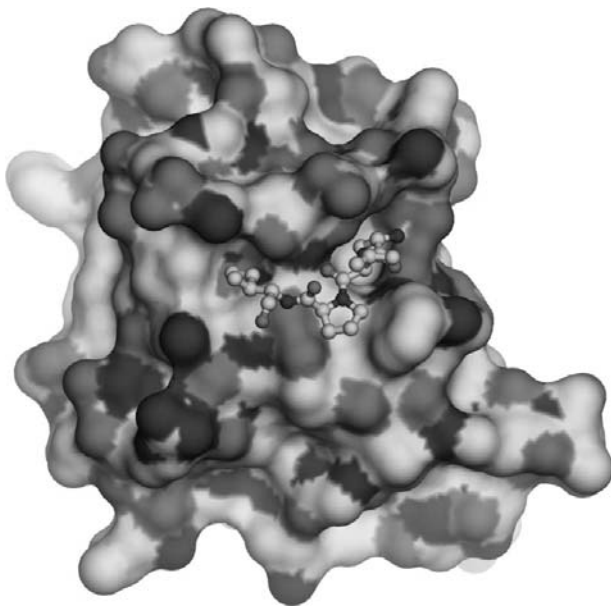


Figure 2-4. Structure of the XIAP BIR3 domain complexed with SMAC tetrapeptide. SMAC peptide bound to BIR3 of XIAP. The BIR3 domain of XIAP (shown as a space-filling model) complexed with the SMAC tetrapeptide, AVPI. See Color Plate 3.

reported inhibitory constants (K_i values) for the eight mammalian members of the IAP family are provided in Table 2-2. The interaction of Survivin with caspases may require additional accessory proteins and post-translational modifications.

6. IAPs AS E3 LIGASES

Several of the IAP family members (XIAP, c-IAP1, c-IAP2, ML-IAP, and ILP-2 in mammals) contain a C-terminal RING domain that binds ubiquitin-conjugating enzymes (E2), endowing them with E3 ubiquitin ligase activity. The types of ubiquitin modifications that IAPs induce on their substrates may vary, with K48-linked polyubiquitin chains representing the best documented and the modification typically associated with targeting for proteasomal degradation. However, some IAPs may also mediate non-degradative ubiquitinylation of substrates (involving K63-linked polyubiquitin chains), such as the receptor interacting kinase (RIP1) protein by c-IAP1 and c-IAP2. The UBA domain, found in XIAP, c-IAP1, c-IAP2, and ILP-2, binds ubiquitin chains and plays a crucial role in several facets of IAP function related to their E3 ligase activities.

Factors regulating the E3 ligase activity of IAPs are not fully understood. IAPs auto-ubiquitinylate themselves, constituting a mechanism for self-induced destruction. Binding of endogenous antagonists such as “second mitochondria-derived activator of caspases” (SMAC) in mammals and analogous proteins in insects can stim-

ulate the self-directed E3 ligase activity of IAPs, leading to more rapid proteasome-dependent degradation, thereby promoting apoptosis. The interaction of survivin with XIAP has been reported to reduce self-directed ubiquitinylation and thereby result in higher levels of XIAP and protection from apoptosis.

Several substrates of IAP-mediated ubiquitinylation have been identified thus far, and more are being discovered as research advances. Generally, all IAP-interacting proteins are candidates for IAP-promoted ubiquitinylation. Among the unanswered questions is how IAPs choose among various E2s to induce K48-linked versus alternatively (e.g., K63) linked polyubiquitin, with very different consequences for substrate degradation versus activation.

7. IAPs AND SIGNAL TRANSDUCTION

The IAP family plays an important role in the regulation of several signaling pathways, including activation of protein kinases. For example, c-IAP1 and c-IAP2 mediate TNF- α -induced NF- κ B activation through non-degradative, K63-linked polyubiquitinylation of RIP1 via interaction with TNF receptor (TNFR)-associated factors 1 (TRAF1) and 2 (TRAF2). In this regard, it is presumed that c-IAP1 and c-IAP2 partner with nonclassical ubiquitin-conjugating enzymes (E2s) responsible for non-K48-linked polyubiquitinylation of RIP1 (such as UBC13, which mediates K63-linked ubiquitinylation), but firm details are lacking. In contrast to stimulating

Table 2-2. Reported inhibitory constants (K_i values) for the eight mammalian members of the IAP family			
K_i (nM)	Caspase-3	Caspase-7	Caspase-9
NAIP full length	14	50	ND
NAIP BIR3	185	ND	33
c-IAP1 full length	>2,000	>2,000	>2,000
c-IAP1 BIR2	>10,000	>10,000	NI
c-IAP2 BIR3	NI	NI	>5,000
c-IAP2 full length	NI	ND	ND
c-IAP2 BIR2	>5,000	>5,000	NI
c-IAP2 BIR3	NI	NI	>5,000
XIAP full length	< 0.8	< 0.07	210
XIAP BIR2	0.7	0.2	NI
XIAP BIR3	NI	NI	10
Survivin	NI	NI	NI
Apollon	NI	NI	ND
Livin/ML-IAP	NI	NI	3,200
Ts-IAP/ILP-2	NI	NI	752
Note: Some values may require further verification and should be treated only as an indication of what has been reported in the literature. ND, not determined; NI, not inhibited.			

Table 2-3. Human IAP family members and their binding partners

Human IAP(s)	Binding partner(s)	Domain(s) involved
c-IAP1, c-IAP2	TRAF1/TRAF2 Rip2	BIR1
XIAP	TAB/TAK	BIR1
XIAP	Rip2	BIR2
XIAP	ARTS	BIR1
c-IAP1, c-IAP2, XIAP, survivin, Livin, ML-IAP	SMAC	BIR2/3 BIR
c-IAP1, c-IAP2, XIAP, NAIP, Livin	XAF1	BIRs
c-IAP1, c-IAP2, XIAP	Caspase-3, -7, -9	BIR2 or BIR3
c-IAP1, c-IAP2, XIAP	c-RAF	Not determined
Survivin	HBXIP, Crm1, Ran-GTPase	BIR
Survivin	Borealin, INCENP	C-Terminus
Survivin	Aurora B	BIR
c-IAP1, c-IAP2, XIAP, Livin, ML-IAP	UBCs (E2s)	RING
NAIP	Bacterial flagellin	LRRs

the TNFR1→ TNF receptor–associated death domain (TRADD)→RIP pathway for NF- κ B activation, the c-IAP1 and c-IAP2 proteins also ubiquitinyrate the serine/threonine kinase, “NF- κ B-inducing kinase” (NIK). NIK is a protein that controls the non-canonical NF- κ B signaling cascade involving p100/p105 NF- κ B family proteins, which undergo limited degradation to produce p50/p52 transcription factor subunits that partner principally with RelB. The c-IAPs promote the destabilization of NIK (presumably involving K48-linked polyubiquitylation) via proteasomal degradation, thus blunting signaling via the non-canonical NF- κ B signaling pathway. Thus effects of c-IAP1 and c-IAP2 on NF- κ B signal transduction pathways are complex. The first BIR domain of c-IAP1 and c-IAP2 binds TRAF1 and TRAF2, the latter of which is also a RING domain-containing E3 ligase. TRAFs are critical intermediaries in signaling for essentially all TNF family receptors and toll-like receptors (TLRs). TRAF1 and TRAF2 collaborate with TNFRs, but not with TLRs.

XIAP, c-IAP1, and c-IAP2 are involved in controlling the stability of c-RAF kinase, a serine/threonine protein kinase that activates Erk1/2-dependent signaling pathways mediating cell proliferation, differentiation, migration, and survival. Knockdown of these IAPs stabilizes c-RAF protein. In this context, XIAP is indirectly involved in the ubiquitinylation of c-RAF by promoting the association of the ubiquitin ligase carboxy terminal Hsc70-interacting protein (CHIP) to a protein complex that contains c-RAF.

XIAP is involved in NF- κ B and MAPK activation, which is mediated by transforming growth factor β (TGF- β) and the BMPs by direct interaction of the BIR1 domain with TGF- β -activated protein kinase 1

(TAK1) binding protein (TAB1), which in turn activates TAK1 to induce NF- κ B and downstream MAPKs. XIAP, as well as cIAP-1 and cIAP-2 also participate in NLRC1 (NOD1) and NLRC2 (NOD2) signalling to stimulate NF- κ B activation and stress kinase activity. Though mechanistic details are lacking, those IAP-family members bind Rip2, a protein kinase that associates via CARD-CARD interactions with NOD1 and NOD2. It is speculated that the IAPs enable NOD1/NOD2 signaling, either by recruiting the TAB/TAK complex directly (XIAP binds TAB/TAK) or indirectly by catalyzing K63-linked polyubiquitination a post-translational modification that binds TAB.

In the context of Survivin's role in mitosis, it is essential in providing proper localization of the chromosomal passenger proteins INCENP, Borealin, and Aurora B, thus ensuring that the kinase Aurora B finds its mitotic substrates. Activation of Aurora B requires its autophosphorylation and binding to INCENP, which then allows for association with Borealin and Survivin. Disruptions in this chromosomal passenger complex result in mitotic catastrophe and cell death.

Table 2-3 lists the human IAP family members and their associated binding partners involved in various signaling pathways.

8. IAP-IAP INTERACTIONS

Several IAP family proteins are capable of forming homo- or hetero complexes that contribute to their functional properties. Survivin, for example, forms homodimers, assisted by a coiled-coil domain located downstream of its BIR. The three-dimensional (3D) structures of the Survivin homodimer have been solved, providing

a firm understanding of their structural basis. In addition, Survivin interacts with XIAP, c-IAP1, and c-IAP2, apparently via BIR–BIR interactions. The association of survivin with XIAP has been reported to reduce auto-ubiquitinylation of XIAP, thus causing accumulation of XIAP and enhancing apoptosis resistance. In another example of a BIR–BIR interaction, XIAP homodimerization via its BIR1 domain has been cited as a crucial event for NF- κ B activation mediated by XIAP. To date, the structures of BIR–BIR complexes have not yet been solved; therefore, the molecular basis for this type of interaction and firm insights are lacking in terms of which BIRs are capable of associating. RING–RING domain interactions among IAP family members have also been reported in the case of XIAP and c-IAP1, where they have been suggested to stimulate ubiquitinylation of XIAP and its subsequent degradation via the proteasome. Further details are needed about the structural basis and functional consequences of IAP–IAP interactions.

9. POST-TRANSLATIONAL MODIFICATIONS OF BIR PROTEINS

At least three types of post-translational modifications of IAPs contribute to their biological roles: ubiquitinylation, proteolysis, and phosphorylation. With respect to phosphorylation, examples of regulatory phosphorylation events have been identified thus far for XIAP and Survivin. Akt/protein kinase B (PKB) is a member of a family of phosphatidylinositol 3-OH-kinase (PI-3K)–regulated serine/threonine kinases that promote cell survival and suppress apoptosis. XIAP is phosphorylated by activated Akt at Ser87, preventing both auto-ubiquitinylation and cisplatin-induced ubiquitinylation of XIAP. Because Akt is hyperactive in many cancers, this post-translational modification may be a common mechanism contributing to tumor cell survival. The cellular functions of Survivin are regulated by multiple kinases, including Cdk1, p34 Cdc2/cyclin B1, Aurora B, and protein kinase A (PKA). Phosphorylation of Survivin on Thr34 by Cdk1 stabilizes Survivin from proteasomal degradation. The mitotic kinase p34 Cdc2/cyclin B is among the kinases capable of Thr34 phosphorylation of Survivin, an event required for at least some of the functions of Survivin as a regulator of cell division. The molecular events reportedly regulated by Thr34 phosphorylation include (1) association with caspase-9 and hepatitis B virus X-interacting protein (HBXIP) for apoptosis suppression; and (2) association with Cdk1 to stabilize Survivin during prometaphase and metaphase. Cyclic AMP (cAMP)–dependent PKA

phosphorylates Survivin at Ser20, resulting in loss of binding to XIAP and other potential cofactors. This phosphorylation appears to occur exclusively on cytosolic pools of Survivin. The Aurora B protein kinase is capable of phosphorylating Survivin at Thr117 during mitosis. However, this phosphorylation event has a negative effect on Survivin and its function as a regulator of cell division. Dephosphorylation of Thr117 on Survivin is required for chromosome orientation and centromere stabilization. It seems likely that additional examples of regulation of IAP family proteins by phosphorylation will eventually be revealed.

With respect to ubiquitinylation, the polyubiquitinylation of IAPs has been alluded to earlier in this chapter as a means of controlling cellular levels of IAPs. Monoubiquitinylation of XIAP has also been reported to regulate the subcellular distribution of XIAP in neurons. Interestingly, the yeast Bir1p protein undergoes modification with small ubiquitin-like modifier (SUMO), a ubiquitin-like protein. SUMO modification is lost in Bir1p variants lacking the BIR repeats.

Regarding proteolysis, XIAP is cleaved by caspases, separating the BIR1-2 region from the BIR3-RING segment of the protein. The functional consequence of this caspase-mediated cleavage event is probably to eliminate XIAP as a barrier to apoptosis.

10. ENDOGENOUS ANTAGONISTS OF IAPs

In mammals, several endogenous antagonists of IAPs have been identified, including SMAC (Diablo), HtrA2 (Omi), apoptosis-related protein in the TGF- β signaling pathway (ARTS), and XAF-1. The SMAC and “high-temperature requirement serine protease” (HtrA2) are both targeted to the mitochondria by an N-terminal targeting sequence. Once inside these organelles, the targeting sequence is cleaved off, revealing a new N-terminus containing an IBM. In SMAC, this sequence is the tetramer Ala-Val-Pro-Ile, whereas in HtrA2, it is Ala-Val-Pro-Ser. SMAC and HtrA2 are released from the intermembrane space of mitochondria in response to apoptotic signals. SMAC is an elongated dimer for which the N-terminal Ala is essential for simultaneous binding to the IBM-binding grooves of BIR2 and BIR3 of XIAP and c-IAP1. (A 3D structure of the XIAP BIR3 domain binding the SMAC tetrapeptide AVPI is shown in [Figure 2-4](#).) On binding, SMAC releases active caspase-3, -7, and -9 from XIAP, thus enabling apoptosis. HtrA2, a hexamer, acts in a similar manner as SMAC binding to displace caspases, but also possesses a serine protease activity that can induce cell death via a non-caspase-dependent mechanism. Recently, other mitochondrial proteins with

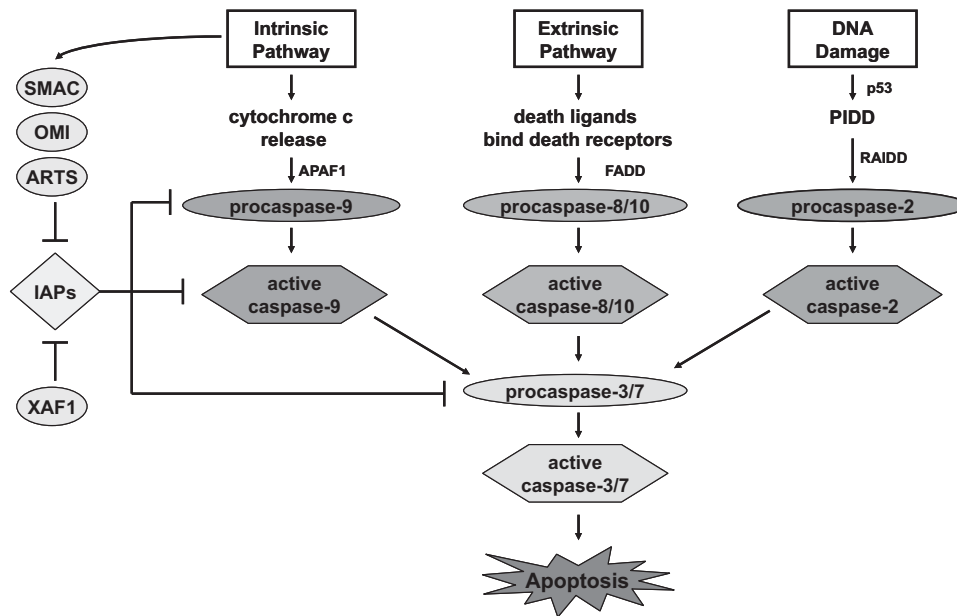


Figure 2-5. IAPs prevent apoptotic cell death. Schematic of human IAPs and caspase inhibition. High levels of IAP lead to caspase inhibition and prevent apoptosis. Interactions with SMAC/Diablo and/or HtrA2/Omi can prevent IAP-mediated inhibition of caspases.

N-terminal IBMs have been identified that seem to primarily target BIR2 of XIAP, including NipSnap (Nsp) 3 and 4, glutamate dehydrogenase (GdH), leucine-rich pentatricopeptide (LRPPR), and 3-hydroxyisobutyrate dehydrogenase (3HB) and other proteins. Several of these proteins (GdH, Nsp4, and LRPPR) have been shown to antagonize XIAP inhibition of caspases-3 in vitro, although not as potently as SMAC. The importance of these other IBM-containing proteins requires additional experimentation. An additional IAP antagonist (GstPT) is processed in the endoplasmic reticulum to remove an N-terminal leader and reveal an IBM, but the conditions that would permit its release from this organelle are unclear.

ARTS is a septin-like protein that resides in the mitochondria; it is released from these organelles and targets XIAP (Figure 2-5). ARTS lacks an IBM sequence and seems to interact with XIAP via a short C-terminal sequence, although other regions of the ARTS protein may also make contact in as much as the GTP-binding domain of ARTS is also required. When released from mitochondria, ARTS is reported to initially colocalize with XIAP initially in the cytoplasm, subsequently accumulating in the nucleus. The half-life of ARTS is regulated by ubiquitin-dependent mechanisms that appear to vary with apoptotic stimuli. Moreover, binding of ARTS to XIAP results in a decrease in XIAP in a proteasome-dependent manner, suggesting that ARTS may participate in controlling XIAP ubiquitinylation. Recently, ARTS was reported to bind and E3 ligase (SIAH,

“seven in absentia”), thus assisting with targeting of XIAP for K48-linked ubiquitination and proteasomal degradation.

XIAP antagonist factor-1 (XAF-1) is another endogenous inhibitor of XIAP. Its mechanism of antagonism seems to involve binding XIAP to induce its shuttling from the cytosol (where caspases reside) into the nucleus, thus effectively separating XIAP from the cellular compartment required for apoptosis suppression. Expression of XAF-1 is significantly reduced in cancer cell lines and primary tumors, apparently as a result of promoter hypermethylation. Additionally, XAF-1 promotes the degradation of Survivin, suggesting a role in both apoptosis and cell division.

In *Drosophila*, multiple IBM-containing proteins have been identified, including Reaper (Rpr), head involution defective (Hid), Grim, Sickie (Skl), and Jafrac2. These proteins function to release DIAPs from the *Drosophila* caspases, *Drosophila* Nedd-2 like caspase (DRONC; initiator caspase) and DCP-1 (effector caspase). In doing so, they contribute to programmed cell death during fly development. Several of the *Drosophila* IAP antagonists have also been reported to stimulate ubiquitin-mediated destruction of IAPs. The N-terminal methionine of Rpr, Hid, Grim, and Skl is removed by an endogenous exoprotease, thus revealing the conserved alanine that initiates the tetrapeptide IBM sequence. The activity of these IAP antagonists appears to be controlled predominantly at the level of gene transcription or mRNA stability via diverse mechanisms that

include participation of regulatory microRNAs in some cases. Jafrac-2, in contrast, is a thioredoxin peroxidase found in the lumen of the endoplasmic reticulum. The N-terminus of Jafrac-2 is proteolytically removed upon import into the endoplasmic reticulum, thus exposing the IBM. 3D structures of *Drosophila* IBMs bound to BIRs of DIAPs have demonstrated an evolutionarily conserved mechanism of molecular recognition that is highly similar to that of their mammalian counterparts.

Chemical compounds have been generated with intent to mimic the tetrapeptide sequences of SMAC and related IAP antagonists for use as apoptosis sensitizers for cancer treatment. Both monovalent and bivalent compounds have been described, the latter binding two adjacent BIRs on target proteins such as XIAP (e.g., BIR2 and BIR3). Compounds with nanomolar potency have advanced to human clinical trials.

11. IAPs AND DISEASE

Somatic mutations and hereditary polymorphisms in IAP family genes have been associated with diseases. For example, chromosomal translocations involving c-IAP2 are found commonly in patients with non-Hodgkin's lymphomas that arise at mucosal locations (mucosa-associated lymphoid tissue [MALT] lymphomas, or MALTomas). These translocations fuse portions of the *c-IAP2* gene on chromosome 11 with portions of the *MALT/paracaspase* gene on chromosome 18, resulting in dysregulation of the signal transduction and E3 ligase activities of c-IAP2. Dysregulation of NF- κ B signaling by c-IAP2/MALT oncoproteins requires TRAF2 binding to the BIR1 domain of c-IAP2. Moreover, amplification of the gene locus that contains *c-IAP1* and *c-IAP2* (which are located adjacent to each other in the human genome) has also been described in lung, cervical, and esophageal cancers.

Humans with X-linked lymphoproliferative disorder (XLP) have been identified with *XIAP* gene mutations that lead to defective expression of this IAP family member. Lymphocytes from patients with XLP have no detectable XIAP expression and demonstrate enhanced apoptosis in response to various apoptotic stimuli. The lymphoproliferative (rather than immunodeficient) phenotype seen in XIAP-deficient XLP patients appears, in part, to result from the observed low numbers of natural killer T-lymphocyte (NKT) cells, suggesting that XIAP may have a role in promoting NKT cell development or survival in humans.

Polymorphisms in the *NAIP* gene on human chromosome region 5q13.1 were initially linked to spinal muscular atrophy, a degenerative disease of spinal motoneu-

rons. However, more recent data suggest that the adjacent and partially overlapping *SMN* gene may be the culprit. In mice, polymorphisms in the *Naip* locus are associated with differential sensitivity to certain types of intracellular bacteria, particularly *Legionella pneumophila*, the cause of Legionnaires' disease in humans. Most inbred strains of mice are resistant to infection, correlating with the non-permissiveness of macrophages to *Legionella* replication. It remains to be determined whether polymorphisms of the *Naip* gene in humans similarly impact susceptibility to bacterial infections.

In addition to polymorphic variants and gene mutants, altered expression of certain IAPs has been associated with diseases in the absence of any known change at the DNA level. In this regard, several IAPs become over-expressed in human cancers, sometimes correlating with clinical outcome. Expression of Survivin is frequently deregulated in cancer, representing one of the most common expression differences observed between malignant and normal tissues. Normally, Survivin expression is limited to fetal and embryonic tissues and is undetectable in fully differentiated adult tissues. Pathological over-expression of Survivin has been reported in most cancers, including breast, colorectal, esophageal, gastric, bladder, ovarian, stomach, pancreatic, liver, and uterine carcinoma, as well as lymphoma and neuroblastoma. High levels of Survivin protein in colorectal and esophageal cancer and glioma are associated with poor clinical outcome, treatment failure, or risk for relapse after therapy. Similarly, over-expression of various members of the IAP family has been documented in a variety of cancers and leukemias, including acute myelogenous leukemias (AML), renal cell carcinoma, prostate cancer, ovarian cancer, colon cancer, melanoma, and non-small-cell lung cancer. In some cases, expression of IAP family members such as XIAP, c-IAP1, or c-IAP2 has been correlated with differences in patient response to chemotherapy or patient survival. Significantly, more than one IAP may be over-expressed simultaneously in cancers, an observation with important implications for developing therapeutic strategies based on IAP antagonism.

Experimentally reducing (knockdown) IAP expression in various cultured cancer cells and tumor cell lines has provided evidence of their importance in sustaining survival of several types of malignant cells in some contexts. In vitro experiments with peptidyl inhibitors of IAPs based on endogenous antagonists such as SMAC and synthetic compounds that attack IAPs have also helped to validate certain members of the family as cancer drug discovery targets. Preclinical animal studies have suggested favorable therapeutic index and have

demonstrated in vivo antitumor activity for such agents. To date, several SMAC-mimicking small-molecule compounds and two AS-ODN drugs are in human clinical trials for cancer.

In addition to cancer, in which IAP expression is commonly elevated, changes in expression of specific IAPs have been linked to neurodegenerative diseases. In Alzheimer's disease (AD), expression of NAIP is decreased in cells taken from the human entorhinal cortex and inversely correlated with paired helical filament-11 (PHA-1) levels, a marker of neurofibrillary tangle pathology. The expression of XIAP is reportedly increased rather than decreased in human entorhinal cortex and hippocampal regions of AD patients, suggesting an attempted compensatory mechanism for surviving AD pathology. Experimental elevations in the expression of NAIP, XIAP, c-IAP1, or c-IAP2 protect neonatal motor neurons from axotomy. The ratio between XIAP and XAF-1 has been correlated with adult motor neuron protection and neonatal neuron sensitivity to axonal injury. NAIP and XIAP both appear to be necessary for preservation of motor neuron survival in rats after sciatic nerve axotomy. Interestingly, expression of XIAP increases in neurons after sciatic nerve injury, suggesting that it contributes to an endogenous defense mechanism. Studies in which neurons were microinjected with cytochrome c have demonstrated an important role for XIAP in preservation of cell survival. These and other related studies have implied that in postmitotic neurons, survival is possible even after mitochondria discharge their contents, provided that IAPs are available to suppress caspases.

Overall, our knowledge of the roles of IAPs in disease remains very limited. Subsequent chapters provide additional details about what is known thus far.

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3

Death Domain–Containing Receptors – Decisions between Suicide and Fire

Henning Walczak and Chahrazade Kantari

1. INTRODUCTION

The tumor necrosis factor (TNF) was among the first cytokines to be characterized both functionally and molecularly. This is due to its pivotal role in physiology and also in pathological conditions. A major driving force behind the initial studies into TNF and its functions was the hope its name already spells out: could TNF serve as a new drug to treat cancer? However, in the end TNF inhibition turned out to be an extremely successful novel therapeutic concept that has revolutionized the treatment of inflammatory diseases such as rheumatoid arthritis, Crohn's disease, and psoriasis, a truly remarkable turnaround.

This turnaround, however, brings up the question of whether there is a molecular basis for what appears to be a misconception at the onset of TNF research. Or is it possible that there is no misconception, but that the two apparently contradictory outcomes are merely the result of different functions of a versatile cytokine? The answer lies in the molecular understanding of the signaling pathways triggered by TNF itself, as well as by the other members of a family of proteins we today refer to as the TNF superfamily (TNFSF) of cytokines. The two fundamental cellular responses triggered by this family of cytokines (i.e., the induction of cell death by apoptosis and the triggering of inflammation) are encrypted in these pathways. They are triggered when TNFSF cytokines cross-link their cognate receptors. By examining the molecular signaling pathways engaged by receptors capable of inducing cell death, we shed light on the differences and similarities between the signals that lead to these diametrically different outcomes.

Receptors capable of inducing cell death by apoptosis form part of the TNF receptor superfamily (TNFRSF). Membership in this protein family is endowed by presence of up to six repeats of a characteristic cysteine-rich domain (CRD) in the extracellular portion of the receptor. These CRDs are crucial for each receptor's binding of its cognate ligand. TNFRSF members are receptors that are able to induce a variety of biological functions after cross-linking by their respective ligands. The versatility of their response spans from the induction of cell death by apoptosis to proliferation and from down-modulation of an immune response to immunostimulatory, proinflammatory action. Most members of the TNFRSF are type I transmembrane proteins, but some are only attached to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. Other TNFRSF members are secreted soluble molecules, acting as decoys for their respective ligands. To date, the TNFRSF consists of 30 members, and the death receptors form a subgroup of this family.

Death receptors are characterized by the presence of an intracellular death domain (DD), a stretch of approximately 80 amino acids that adopts a characteristic conformation, now generally referred to as the DD fold. To date, six human DD-containing receptors have been identified: TNF-R1 (p55/p60 TNF-R), CD95 (Fas, APO-1), death receptor 3 (DR3, TRAMP), TRAIL-R1 (DR4), TRAIL-R2 (DR5), and DR6 (TNFRSF21). These receptors are activated by their respective ligands: TNF, CD95L (FasL/APO-1L), TL1A, TRAIL (Apo2L), and, as recently suggested, a specific amino-terminal cleavage fragment of the β -amyloid precursor protein (APP), N-APP (Table 3-1 and Figure 3-1). As expected, the DD plays a crucial role in signaling induced by these receptors as

Table 3-1. The six human DD-containing receptors and their ligands	
Receptor(s)	Ligand(s)
TNF-R1 (p55/p60 TNF-R)	TNF lymphotoxin- α (LT- α)
CD95 (Fas/APO-1)	CD95L (FasL/APO-1L)
DR3 (TRAMP)	TL1A
TRAIL-R1 (DR4)	TRAIL (Apo2L)
TRAIL-R2 (DR5)	
DR6	
	APP (not a member of the TNFSF; possibly another ligand is still not identified)

it enables the recruitment of proteins that themselves contain a DD. The most prominent and decisive integrators of death receptor signaling are the proteins known as Fas-associated DD (FADD or MORT1) and TNFR-associated DD (TRADD). FADD and TRADD are adaptor proteins. This means they themselves do not exert any enzymatic function but instead act by forming a bridge between proteins, in this case between receptor and signaling effector proteins. Dependent on whether a death receptor recruits FADD or TRADD to its DD, it can be categorized into one of two classes: although cross-

linking of CD95, TRAIL-R1, and TRAIL-R2 results in the recruitment of FADD, TRADD is recruited to ligand-activated TNF-R1 and DR3. The FADD-recruiting class of death receptors induces apoptosis as their primary signaling output, whereas the TRADD binds primarily induce immunostimulatory, proinflammatory signaling. At this time the only DD-containing receptor that does not fall into any of these two categories is DR6. It will be interesting to discover which third kind of signaling may be triggered by this receptor. A first hint comes from the recent identification of N-APP as a DR6 ligand.

In the following sections, we cover the five different death receptor–ligand systems. We first address the two FADD-recruiting receptor–ligand systems, the CD95 and the TRAIL systems. In this part we also make reference to some of the historical landmark findings in apoptosis research originating from the study of these systems. In the following section, we discuss the TRADD-recruiting immunostimulatory, proinflammatory TNF-R1 and DR3. Then we examine the current knowledge on DR6 before we finally juxtapose the signaling systems of FADD- and TRADD-binding receptors to define the similarities and differences between the two main types of signaling triggered by DD-containing receptors.

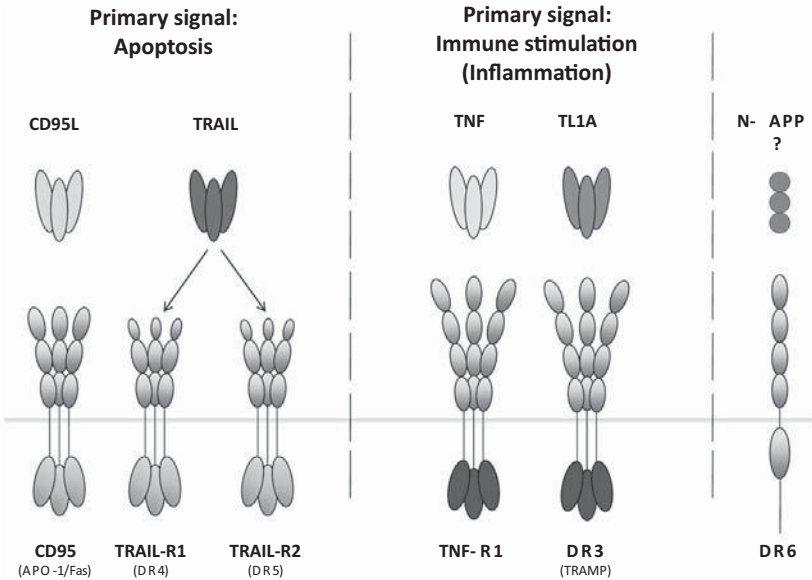


Figure 3-1. The human death domain (DD)-containing receptors and their known ligands. The six human death domain (DD)-containing receptors are transmembrane proteins that contain repeats of two to four cysteine-rich domains (CRDs) in the extracellular portion required for the ligation of their cognate ligand and an intracellular DD capable of recruiting adaptor proteins that will trigger downstream intracellular signals (light gray for CD95/TRAIL systems and dark gray for TNF-R/DR3 systems). DD-containing receptors mainly trigger two signals: CD95, TRAIL-R1, and TRAIL-R2 interact with the adaptor protein FADD and therefore induce apoptosis as their primary signaling output, whereas TNFR-1 and DR3 interact with TRADD and hence mainly trigger proinflammatory signals. The molecular events that initiate DR6 signaling have not been elucidated.

2. RECEPTOR-LIGAND SYSTEMS WITH PRIMARILY PROAPOPTOTIC FUNCTIONS

2.1. The CD95 (Fas/APO-1) system

2.1.1. CD95 and CD95L: discovery of the first direct apoptosis-inducing receptor-ligand system

In 1989, two monoclonal antibodies, anti-APO-1 and anti-Fas, were described. Although anti-APO-1 was reported to bind to a cell surface protein of approximately 48 kD, anti-Fas was thought to bind to a cell surface receptor of approximately 200 kD, suggesting that APO-1 and Fas would be two different antigens. Both anti-APO-1 and anti-Fas rapidly induced cell death in a variety of human cancer cells by a then very poorly understood cellular process known as apoptosis. The systematic and specific employment of these two antibodies in the following decade most likely uncovered more mysteries and contributed more to our understanding of apoptosis than any other approach, possibly only matched on the antiapoptotic side by the study of Bcl-2.

The first question addressed by the groups working with anti-APO-1 and anti-Fas was to which receptors the respective antibodies bind. Whereas the group led by Shigekazu Nagata in Osaka, Japan, performed an expression cloning strategy with anti-Fas, Peter Krammer's team in Heidelberg, Germany, pursued a classical purification approach with anti-APO-1. The surprising result was that, despite the indications of the original biochemical characterization, anti-Fas and anti-APO-1 recognized the same antigen. They both bound to a cellular receptor of 48 kD now commonly referred to as CD95 or Fas (APO-1) (Itoh et al., 1991; Oehm et al., 1992). The cloning of CD95 revealed that it contained three cysteine-rich domains (CRDs) in its extracellular portion, qualifying it as a new addition to the TNFRSF, which at the time only consisted of a handful of members and was not yet referred to as a superfamily for exactly this reason. The functional dissection of the intracellular domain of CD95, on the other hand, revealed the presence of a discrete domain that was required for induction of cell death. This domain is now known as the previously mentioned death domain (DD). Employment of the DD of CD95 in a yeast two-hybrid screen by the groups of Vishva Dixit in Ann Arbor, Michigan, and David Wallach in Rehovot, Israel (Boldin et al., 1995; Chinnaiyan et al., 1995), led to the discovery of FADD (MORT1), a protein that contained a DD and a second, DD-like domain, which is now known as a death effector domain (DED).

At this point, it remained a mystery how binding of FADD to CD95 induced the drastic biochemical changes characteristic of apoptosis. However, the discovery of the death-inducing signaling complex (DISC), induced when anti-APO-1 cross-links CD95, illuminated the picture. Kischkel et al. found that receptor cross-linking resulted in recruitment of FADD and two other proteins, as revealed by two-dimensional (2D) gel electrophoresis of the protein complex that forms when CD95 is cross-linked by anti-APO-1 (Kischkel et al., 1995). The two spots turned out to be processed and unprocessed forms of the same enzyme. This enzyme was first named FLICE, for FADD-like ICE (interleukin [IL]-1-converting enzyme), or MACH, and it was codiscovered by the Wallach team and by a concerted effort of the Dixit and Krammer teams. FLICE/MACH is now known as caspase-8. Caspase-8 is present in the cytosol as a proenzyme. It is activated by a conformational change induced by FADD-mediated recruitment to CD95. Caspase-8 activation at the CD95 DISC then triggers a caspase cascade, which induces the execution of the apoptotic cell death program. The identification of caspase-8 recruitment and activation at the DISC provided the missing link between extracellular cross-linking of a receptor and intracellular activation of an enzymatic event, which triggers an entire proteolytic cascade responsible for all the biochemical hallmarks of apoptosis. This transition from the outside to the inside of the cell possibly resembles one of the most striking and beautiful examples of energy translation across a membrane in cell biology.

An entirely different line of research was opened when it was found by the Nagata team that murine CD95 was mutated in mice, which had long been studied as a model system for lupus erythematosus. These mice, known as *lpr* mice, carried a mutation in the murine CD95 gene that interfered with its proper expression. As a result, these mice exhibit a massive accumulation of lymphocytes. This had been mistaken for lymphoproliferation (*lpr*) due to the fact that, before the advent of apoptosis research, scientists automatically assumed that accumulation of cells would be due to abnormally high proliferation rather than a block in cell death. However, it turned out that T cells in *lpr* mice have a defect in cell death and that they therefore accumulate. A similar phenotype had been observed in *gld* (generalized lymphoproliferative disease) mice and elegant bone marrow transplant experiments by Cohen and Eisenberg (Chapel Hill, North Carolina) had suggested that the genes mutated in *lpr* and *gld* mice encoded an interacting pair of proteins on interacting cells (Cohen et al., 1992). When the CD95 ligand (CD95L/FasL/APO-1L) was then cloned by the Nagata team, this time

employing both a purification and an expression cloning approach, this prediction turned out to be true: the gene encoding CD95L is mutated in *gld* mice. However, this finding did not yet explain the biological phenomenon responsible for the *lpr* and *gld* phenotypes. This was solved when the Krammer team and two other groups led by Ann Marshak-Rothstein (Boston, Massachusetts) and Douglas R. Green (San Diego, California), respectively, discovered that the interaction between CD95 and CD95L is responsible for a major portion of activation-induced cell death (AICD) in T cells (Ju et al., 1995; Dhein et al., 1995; Zhang et al., 1997). Subsequently, CD95L was also found to be responsible for the long-known perforin-independent killing activity of CD8⁺ cytotoxic T cells. Thus the CD95 system does not only play a role in the homeostatic regulation of the immune system, but is also employed in the defense mechanisms used by the immune system to fight infection.

When research into the CD95/CD95L system began, it was hoped that agonists of CD95 would hold the promise that TNF unfortunately could not keep as a result of the detrimental effects associated with its systemic application. These hopes were, however, immediately crushed to pieces when it became clear that animals systemically treated with antibodies to CD95 or with recombinant CD95L died within hours due to fulminant hepatitis.

As disappointing as this outcome first appeared at the time of its discovery, it sparked another line of research. Apparently, the CD95L had an enormous capacity to harm normal human tissue. It was, however, not known to what extent this function was actually involved in various pathological conditions in which apoptotic damage occurred to normal tissue. Thus began the investigation of the role of the CD95/CD95L system in various diseases in which normal tissue is damaged. In the meantime, there is ample evidence for the involvement of the CD95 system in various diseases, including very strong evidence for its involvement in graft-versus-host disease and some forms of acute hepatitis, but also data that indicate a role for this system in acute myocardial infarction, stroke, and spinal cord injury, among others. An additional and rather unexpected, but potentially quite broad, application of CD95L inhibitors was suggested recently when it was shown that instead of inducing apoptosis, CD95 can also induce migration in certain cancer cells, and that inhibition of CD95L was able to halt this effect. Although we are only beginning to understand the molecular mechanisms of this type of signaling induced by CD95L-mediated stimulation of CD95, its elucidation will be of pivotal importance to identify markers for those types or individual cases of cancer that

may respond to a CD95L-inhibiting therapy by exhibiting less migration and hence a reduction in metastasis. Recently, the first biotherapeutic inhibitor of CD95L, a soluble CD95-Fc fusion protein, successfully passed phase I clinical testing. It will be interesting to see how its further clinical testing will unfold. Therefore, it seems that for the CD95 system, as in the case of TNF (which is covered later in this chapter), the most prominent medical applications will most likely derive from antagonizing rather than stimulating it. It should, however, be mentioned that there are attempts to also use certain recombinant forms of CD95L as cancer therapeutics. Obviously such trials must be carried out with extreme care not to harm any tissues, especially the liver.

2.1.2. Biochemistry of CD95 apoptosis signaling

CD95 is ubiquitously expressed, though predominantly in the thymus, liver, heart, and kidney. In contrast, CD95L expression is very restricted: it is primarily expressed by activated T cells. Even if CD95 and CD95L are mostly expressed as membrane-bound proteins, soluble forms of both receptor and ligand also have been reported. The exact roles of soluble CD95 and CD95L are still unclear. However, soluble CD95 is thought to counteract CD95-induced apoptosis, whereas soluble CD95L, generated by metalloprotease-mediated cleavage, has been shown to either possess killing activity or to act as an inhibitor of membrane-bound CD95L, the outcome depending on how the signaling events triggered by receptor cross-linking are integrated in the particular target cell. Even though to date the CD95 system is the best characterized direct apoptosis-inducing receptor-ligand system, it can also trigger other signaling outcomes, ranging from proliferation to proinflammatory signaling and even to increased motility. However, the induction of apoptosis remains its most prominent function.

CD95-induced apoptosis is triggered when CD95L-mediated cross-linking of CD95 receptors, preassembled on the surface of the cell, leads to the recruitment of the adaptor protein FADD, the proteases caspase-8 and caspase-10, and the cellular FLICE-like inhibitory protein (cFLIP). Together, these proteins constitute the DISC. The exact molecular mechanism of CD95 activation by its ligand-induced cross-linking has only recently been uncovered. In a first step, stimulation of CD95 by its ligand results in the stabilization of an open conformation of the intracellular domain (ICD) of CD95. This open conformation contains two newly formed helices: the stem helix, created by the fusion between two helices of CD95, and a C-terminal helix. As a result of

these structural rearrangements, the CD95 ICD can then interact, via weak molecular interactions, with another CD95 ICD brought into close proximity by the trimerized CD95L. Interactions between different CD95 ICDs stabilize the open conformation and facilitate the recruitment of adaptor molecules.

FADD is recruited by a homotypic interaction between its DD and the DD of the CD95 ICD. The following recruitment of the initiator caspase-8 and -10 again requires homotypic interactions, this time between the respective DEDs of FADD and the caspases. We describe the process leading to the activation of caspase-8 because it is virtually identical to that of caspase-10. Homotypic interaction of the DEDs of FADD and caspase-8 results in dimerization of caspase-8. Recruitment and dimerization induce a conformational change that allows caspase-8 to become enzymatically active. It is important to note that it is not the cleavage of caspase-8 that activates it, but rather the conformational changes induced by its recruitment to the DISC and the juxtaposition with a second caspase-8 monomer at this protein complex. Active caspase-8 then cleaves itself, but most importantly, it proteolytically activates the downstream effector caspase-3. These effector caspases then perform the proteolysis of vital cellular proteins, including structural components such as lamins and gelsolin, but also other proteins such as poly(ADP)-ribose polymerase and the inhibitor of caspase-activated DNase. The latter event liberates the caspase-activated DNase from cytosolic retention and thereby allows for one of the hallmarks of apoptosis, the cleavage of nuclear DNA, to take place. The proteolysis of effector caspase substrates is responsible for the characteristic biochemical and morphological hallmarks of apoptosis. The antiapoptotic factor cFLIP can prevent CD95-induced apoptosis at the level of caspase-8. This protein is structurally similar to caspase-8 and -10 as it contains two tandem N-terminal DEDs. However, unlike these cysteine proteases, it lacks a cysteine in what otherwise would be its active center. Hence cFLIP lacks enzymatic activity as a protease. Three different splice variants of cFLIP have been described – cFLIP_L, cFLIP_S, and cFLIP_R – and they may exert their inhibitory effects on CD95-induced apoptosis differentially.

The proapoptotic BH3-only family member Bid is a critical substrate of caspase-8 and -10. Caspase-8/10 cleaved, truncated Bid (tBid) translocates from the cytosol to the outer mitochondrial membrane where it can induce mitochondrial outer-membrane permeabilization (MOMP) if the molecular composition with respect to other members of the Bcl-2 protein family allows it to do so. These processes are discussed

in detail in other chapters of this book. In the context described here, it suffices to point out that BID and its cleavage by caspase-8 or -10 are what link the death receptor apoptosis pathway with the mitochondrial pathway of apoptosis induction. However, one crucial consequence of activation of the mitochondrial apoptosis pathway can be decisive for the outcome of CD95 stimulation, at least in cells referred to as type II cells. MOMP induces the release of proteins from the mitochondrial intermembrane space. Most importantly, these proteins are cytochrome c as the first caspase-activating factor, but also the second mitochondrial activator of caspases (SMAC), also known as DIABLO. Whereas cytochrome c release triggers the formation of the apoptosome resulting in activation of caspase-9, release of SMAC induces the neutralization of the X-linked inhibitor of apoptosis protein (XIAP). Once XIAP is inhibited by SMAC, caspase-3, -7, and -9, which are all inhibited by XIAP, are released from inhibition, and cell death can finally ensue (Figure 3-2). Thus, in cells that express high levels of XIAP, the direct activation of caspase-3 by caspase-8 is blocked so that these cells require the pro-mitochondrial changes brought about by the cleavage of BID and its proapoptotic activity on mitochondria to succumb when CD95 is activated. It therefore seems that the expression of XIAP as compared with lack thereof explains the dichotomy of cells with respect to their categorization as type I and type II cells for CD95-mediated apoptosis. Differences in the extent of CD95 DISC formation, first thought to be the sole cause for the type I/type II distinction, may contribute to this.

2.2. The TRAIL (Apo2L) system

Although CD95L itself most likely will not become a major drug in cancer therapy, its discovery paved the road to the identification of a new member of the TNF cytokine family. In 1995, two groups, one at Immunex in Seattle, Washington, and one at Genentech in San Francisco, California, independently found that there was an expressed sequence tag (EST) in the public database that was even annotated as being homologous to CD95L. The TNF-related apoptosis-inducing ligand (TRAIL), or Apo2L, as it was named by these two groups, respectively, seemed to specifically kill cancer cells. A number of cancer cell lines were susceptible to TRAIL-induced apoptosis, whereas the normal cells tested were not. So could it be that TRAIL would finally fulfill the hopes placed initially on TNF and then on CD95L? The answer came in 1999 by studies from both the Immunex and the Genentech groups: systemic treatment of tumor-bearing mice with recombinant TRAIL, which,

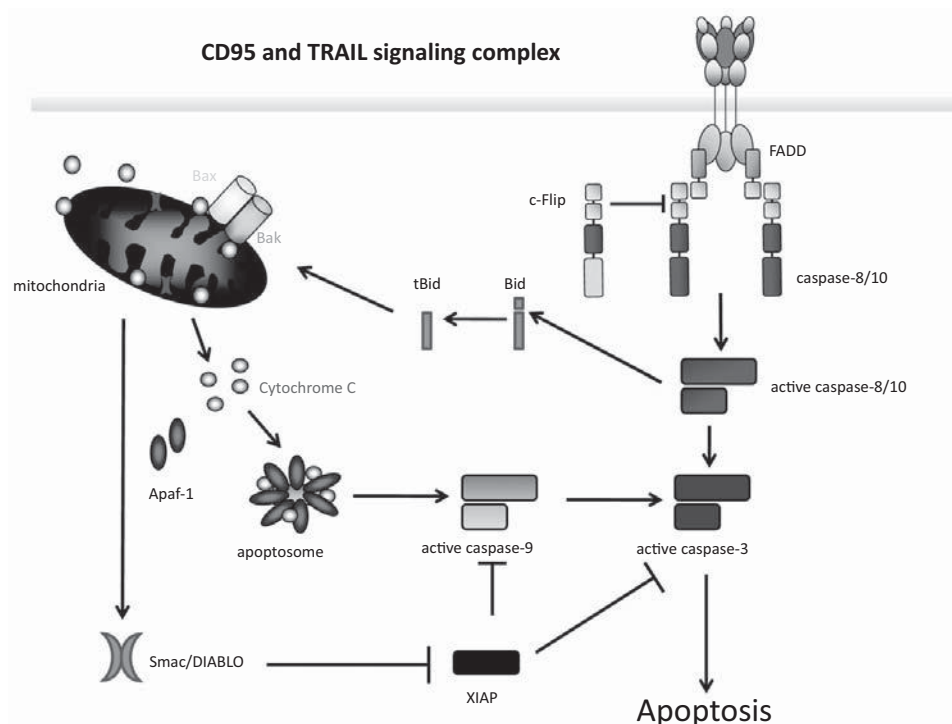


Figure 3-2. Schematic representation of apoptotic signaling by the CD95 and TRAIL systems. Binding of CD95 or TRAIL to their respective receptors leads to receptor trimerization and formation of the death-inducing signaling complex (DISC). The adaptor protein FADD is recruited to the DISC where the death domains (DD) of both proteins interact. Subsequently, procaspases 8 and 10 are recruited to the protein complex where they interact with FADD via the death effector domains (DEDs). cFLIP can compete with caspase-8 for the binding to FADD. Therefore, high levels of cFLIP can abrogate caspase-8 activation at the DISC. DISC-activated caspase-8 and -10 trigger a caspase cascade by cleavage of caspase-3. In addition, Bid is cleaved into tBid, which initiates the mitochondrial apoptosis pathway, leading to release of cytochrome c (CytC) and SMAC/DIABLO from the mitochondria. CytC, together with Apaf-1 and caspase-9, forms the apoptosome, an activation platform for caspase-9. SMAC/DIABLO counteracts the caspase-inhibitory function of XIAP, thereby allowing for full activation of caspase-3 and -9, ultimately leading to cell death. See Color Plate 4.

importantly, was also capable of binding to and killing mouse cells, killed tumor cells *in vivo* without harming normal tissue and thereby ablated tumor growth (Walczak et al., 1999). By demonstrating that a TNF-like cytokine can be used systemically *in vivo* to specifically kill tumor cells, these results represented the culmination of decades of research into the agonistic action of TNF family members.

Given these encouraging results, Immunex and Genentech decided to join forces so that together they would be able to fully explore the clinical potential of this promising new avenue in the treatment of cancer. In the meantime, Apo2L/TRAIL is in various clinical trials, and it is clear from these trials that there is clinical efficacy. However, it is also apparent from these trials that we are only beginning to understand the clinical potential of this drug and, in fact, a whole new class of cancer drugs, which we will refer to as TRAIL receptor agonists. This is partly due to the receptor promiscuity of TRAIL, which we discuss next.

After TRAIL was identified, the race for the cloning of its receptor began. At the time, in 1996 and 1997, many new human genes were either found in the public database of human EST sequences provided by the Human Genome Project or by Human Genome Sciences, a company that had its own private "little" human genome project. However, because it was clear that the apoptosis-inducing TRAIL receptor was going to be very valuable, as antibodies against it would potentially become new cancer drugs, other approaches also were pursued, including expression cloning and purification of the TRAIL receptor. In the end, purification and EST-based techniques were successful. The EST approach was, however, first to discover an apoptosis-inducing receptor for TRAIL (now referred to as TRAIL-R1 or death receptor 4 [DR4]). Yet the purification approach followed only weeks later with the discovery of a different apoptosis-inducing receptor for TRAIL, the receptor now referred to as TRAIL-R2 or DR5. Shortly after that, TRAIL-R2 was also discovered by a number of other groups as

its sequence then appeared in the public and private EST databases only a few weeks after it was characterized and identified by purification.

However, the search for TRAIL receptors was not over yet. In the subsequent months, work by a number of groups led to the identification of two other cell-bound receptors for TRAIL, TRAIL-R3 (DcR1), and TRAIL-R4 (DcR2). These two receptors do not induce apoptosis, and it was first thought by some authors that they may exert a decoy function for TRAIL (hence the name “decoy receptor” [DcR]), which would be particularly expressed by normal cells and responsible for protecting them from TRAIL-induced apoptosis and for TRAIL's tumor-selective killing activity. However, an expression pattern of TRAIL-R3 and/or TRAIL-R4 as being present on normal but not on cancer cells was never found, putting the decoy concept for these receptors into question. Finally, it was found that TRAIL binds to a fifth receptor, osteoprotegerin (OPG). OPG is a soluble TNFRSF member that is mainly described as a regulator of the development and activation of osteoclasts in bone remodeling. It binds TRAIL only with low affinity, and its high-affinity ligand is the TNFSF member RANKL, which, apart from binding to OPG, also binds to the cell surface receptor activator of nuclear factor kappa B (NF- κ B) (RANK), inducing this receptor's osteoclast differentiation activity. It is, however, rather unlikely that the reported interaction of TRAIL with OPG is relevant *in vivo* because mice over-expressing TRAIL do not exhibit any bone-related phenotype, which would have been expected if TRAIL were capable of interacting with the bone-protective OPG *in vivo*.

In summary, TRAIL interacts with five receptors: the four membrane-bound TRAIL receptors, TRAIL-R1 to TRAIL-R4, and the soluble receptor OPG. TRAIL is therefore the most promiscuous of all TNFSF members. The biological basis for this promiscuity is still unclear. Whereas TRAIL-R1 (DR4) and TRAIL-R2 (DR5, Apo-2, KILLER, TRICK2) are DD-containing receptors capable of triggering apoptosis, TRAIL-R3 and TRAIL-R4 cannot do so because of the lack of an intracellular DD.

TRAIL-R1 and TRAIL-R2 share 58% sequence homology, and thus far it has not been possible to identify distinct functions of one receptor versus the other. They both trigger apoptosis via the same pathway, and this pathway is even identical to the one described above for CD95. TRAIL-R3 lacks an intracellular domain and is inserted into the plasma membrane via a GPI anchor. TRAIL-R4 has a cytosolic domain, but there is only a truncated DD of 15 instead of 80 amino acids, which is not capable of inducing cell death. However, TRAIL-R4 can activate NF- κ B. As mentioned above,

TRAIL-R3 and TRAIL-R4 are often referred to as decoy receptors because they were shown in some of the cloning papers to sequester TRAIL on over-expression, thereby inhibiting TRAIL-induced apoptosis. To exert this death-inhibitory effect, TRAIL-R3 and TRAIL-R4 would have to present with a higher affinity for TRAIL or be expressed at substantially higher levels than TRAIL-R1 and/or TRAIL-R2. However, this is not the case. Others have proposed a model in which TRAIL-R3 and TRAIL-R4 interact via a pre-ligand assembly domain to inhibit ligand binding. A third notion suggests that the NF- κ B-inducing activity of TRAIL-R4 may antagonize the death signal. To summarize this, we are still pretty much completely in the dark regarding the actual function of these two receptors in the biology of TRAIL, and not much progress has been made in the understanding of their function since they were cloned more than a decade ago. It will be important to study their function under non-over-expression conditions to uncover their physiologic role in TRAIL biology.

The biggest conundrum is still the difference between the TRAIL and the CD95 system with respect to the differential outcome of the *in vivo* application of agonists to CD95 as compared with agonists of TRAIL-R1 and/or TRAIL-R2. Despite the fact that no significant differences in the signaling pathways triggered by these two systems have been discovered to date, the outcome of their stimulation by systemic application of CD95 versus TRAIL-R1/2 agonists could not be more disparate. It remains one of the mysteries in apoptosis research today what the biochemical basis for this difference is, and it will be highly rewarding to identify its cause because it is likely to open the door to a more targeted application of TRAIL receptor agonists in specific cancer patients or patient groups.

With respect to this novel class of cancer drugs, apart from Apo2L/TRAIL itself, a total of five TRAIL-R2- and one TRAIL-R1-specific monoclonal antibodies are being developed. Many of them are already in various phase II clinical trials for the treatment of different cancer entities. This topic has recently been reviewed elsewhere, and we will therefore not go into further detail here (Johnstone et al., 2008; Papenfuss et al., 2008; Ashkenazi, 2008). Nevertheless, we would like to highlight one important and somewhat troubling aspect of these current trials. The one big absentee from the current clinical trials with TRAIL receptor agonists is a set of biomarkers guiding the selection of specific combinations of drugs that would be most likely to be effective in individual cancer patients who present with a particular genetic make-up of their cancer. To provide such (sets of) biomarkers for future trials and ultimately

for the best possible clinical use of the different TRAIL receptor agonists, it is of pivotal importance that we spend more time and effort on the thorough understanding of the biochemical mechanisms of TRAIL apoptosis resistance versus sensitivity of different types of cancer cells that rely on specific combinations of alterations in the expression of a particular set of oncogenes and tumor suppressor genes as compared with normal cells. Thereby we may be able in the future to specifically target expression or activity of certain factors to achieve cancer-specific TRAIL apoptosis sensitization on the level of the individual cancer patient. Novel proteomic and genomic technologies and the integration of the results obtained by their application in intelligent systems biology approaches will most likely be instrumental in uncovering the mechanisms that govern TRAIL apoptosis sensitivity versus resistance in different types of cancer. These studies will enable a more targeted and individualized use of TRAIL receptor agonists and combination with other drugs in cancer therapy in the future.

3. DEATH RECEPTOR–LIGAND SYSTEMS WITH PRIMARILY IMMUNOSTIMULATORY, PROINFLAMMATORY ACTIVITY

3.1. The TNF system

3.1.1. Biochemistry of TNF signal transduction

The founding member of the TNFSF is a homotrimer of TNF molecules, each 157 amino acids in length. The trimer adopts a characteristic conformation, which is now commonly referred to as the TNF fold. TNF is mainly produced by activated macrophages. Depending on the physiologic or pathological context, it is, however, also expressed by a number of other cell types. The binding of TNF to its receptors triggers a series of intracellular events that primarily induces the activation of NF- κ B and the mitogen-activated protein (MAP) kinases c-Jun N-terminal kinase (JNK) and p38. These events lead to immunostimulatory gene induction, which often drives an inflammatory response (Figure 3-3). Induction of apoptosis by TNF is only a secondary signal (see Section 5).

The diverse biological effects of TNF are mediated by two different receptors, TNF-R1 and TNF-R2. Although TNF-R1 is expressed on cells of almost all tissues, TNF-R2 is almost exclusively present on cells of lymphoid origin. TNF-R1 contains a DD and initiates the majority of TNF-induced biological activities, including induction of cell death by apoptosis. Yet TNF-R2 was also shown to be capable of inducing apoptosis. It has now been demonstrated, however, that TNF-R2–induced apopto-

sis works via an indirect loop mechanism: TNF-R2 cross-linking induces expression of TNF, which then binds to TNF-R1 to induce cell death. Apart from inducing TNF, the TNF-R2–mediated signal also sensitizes cells to TNF-R1–mediated apoptosis by depleting TRAF2 and cIAPs, which causes the gene-inducing capacity of TNF-R1 to be diminished, strengthening the apoptotic arm of the response (Figure 3-4). Thus the TNF-R2 signal is a modulator of the TNF-R1 signal transduction machinery, and other non-DD-containing receptors of the TNFRSF described to induce apoptosis in certain cells, including CD40, CD30, and FN14, also induce TNF and therefore work in a fashion similar to TNF-R2. Because TNF-R1 is the main signaling receptor for TNF, we now examine its activities in more detail. Binding of TNF to TNF-R1 induces receptor oligomerization and recruitment of cytoplasmic signaling proteins, leading to the formation of the TNF-R1 signaling complex (TNF-RSC). The composition of the TNF-RSC and the following steps in TNF-R1–mediated signaling have been extensively studied over the last decade. Although further analysis will be required to discover all the players involved in this process, it is fair to say that to date, it is one of the best understood receptor signaling complexes and cascades in cell biology.

On activation of TNF-R1 by TNF-induced cross-linking at the plasma membrane, the TNFR1 DD serves as a docking site for the DD-containing adaptor protein TRADD. TRADD is recruited to the DD of TNF-R1 via a homotypic DD interaction. TRADD in turn recruits the TNF-R-associated factor-2 (TRAF2) and the receptor-interacting protein 1 (RIP1), a serine/threonine kinase. RIP1, however, can also directly bind to TNF-R1 via its own DD without the need for TRADD. The importance of this interaction remains unclear. Recruitment of TRAF2 (or TRAF5) by TRADD enables recruitment of cIAP1 and/or cIAP2 to the TNF-RSC. The ubiquitin ligase activities of both TRAFs and cIAPs are required for decoration of RIP1 by polyubiquitin chains and for NF- κ B and MAP kinase activation. Ubiquitin chains can be formed via linkages of the ubiquitin subunits on different ϵ -amino groups of the seven different lysines present in ubiquitin or via the α -amino group at the amino-terminus of ubiquitin, with the latter creating linear ubiquitin chains. Thus far it is thought that polyubiquitin chains involved in TNF signaling are either linked via the ϵ -amino groups of lysine 63 (K63) or K48 of ubiquitin. However, recent data obtained by us and others revealed that linear ubiquitin chains also play an important role in this process. A protein complex termed LUBAC (for linear ubiquitin chain assembly complex) forms an integral part of the TNF-R1 signaling complex (Haas et al., 2009). Furthermore, LUBAC is required for

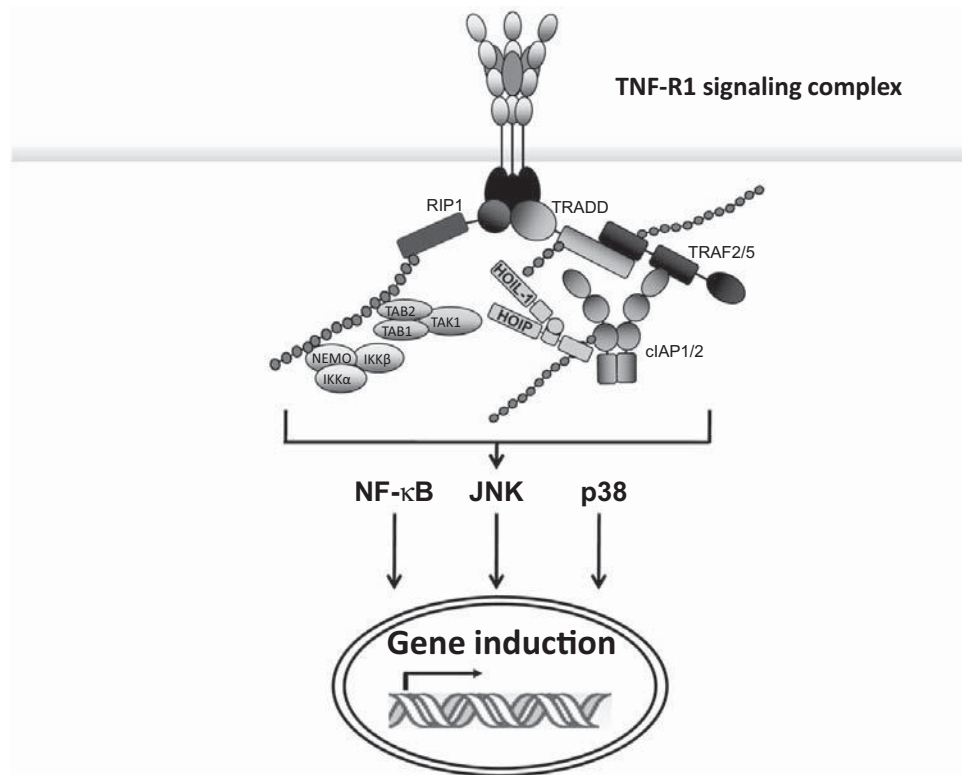


Figure 3-3. Schematic representation of immunostimulatory, proinflammatory signaling by the TNF-R and DR3 systems. Binding of TNF and TL1A to their respective receptors leads to receptor trimerization and formation of a receptor signaling complex. First the adaptor protein TNF-R1-associated death domain (TRADD) is recruited via its DD to the DD of the receptor. TRADD then serves as an assembly platform for binding of TRAF2, cIAP1/2, and the receptor interacting kinase 1 (RIP1). TRAFs and cIAPs conjugate ubiquitin chains to various proteins in the complex, which allows for the recruitment of further signaling proteins, including the TAK/TAB and the IKK complexes, ultimately leading to activation of NF- κ B and the JNK and p38 MAP kinase pathways. See Color Plate 5.

efficient TNF-induced NF- κ B activation because NF- κ B essential modulator (NEMO) binds strongly to linear but only weakly to K63-linked ubiquitin chains. Together, K63-linked and linear polyubiquitination of different components of the TNF-RSC result in stable TNF-RSC formation and thereby enable the events that ultimately lead to activation of NF- κ B and the JNK and p38 MAP kinase pathways. The molecular processes that lead to the activation of these pathways have been extensively reviewed in the past (Hayden and Ghosh, 2008; Wajant et al., 2003). The discovery of LUBAC as a novel integral component of the TNF-RSC and linear ubiquitination as a central player in the organization of this protein complex will undoubtedly substantially affect our current view of how these processes are regulated. It will be exciting to unravel these mechanisms at the molecular level and discover how they control the function of TNF.

3.1.2. TNF and TNF blockers in the clinic

Soon after the isolation of TNF, it became clear that the systemic administration of TNF is highly toxic as

a result of the extreme cytokine production it induces. This inflammation-like syndrome prevented the further development of TNF for systemic use. However, a technique called isolated limb perfusion (ILP) has been developed by Ferdinand Lejeune in Lausanne, Switzerland (Lejeune et al., 1995). ILP facilitates local and exclusive administration of TNF to, for example, an arm or leg of a patient with cancer. ILP with TNF in combination with chemotherapeutic drugs led to complete response rates in some patients with sarcomas and melanomas on extremities and showed improved penetration of the cytostatic drugs melphalan and doxorubicin into tumors in animal models. The possibility to inhibit any leaked TNF in the rest of the body with therapeutic TNF blockers, which are now available, may help to overcome the limitations posed so far on ILP by the detrimental effects of potential TNF leakage. Interestingly, TNF specifically disrupts tumor-supporting blood vessels while sparing normal blood vessels. It would be interesting to examine whether endothelial cells in the tumor-associated neovasculature are particularly sensitive to TNF-induced apoptosis and what the biochemical

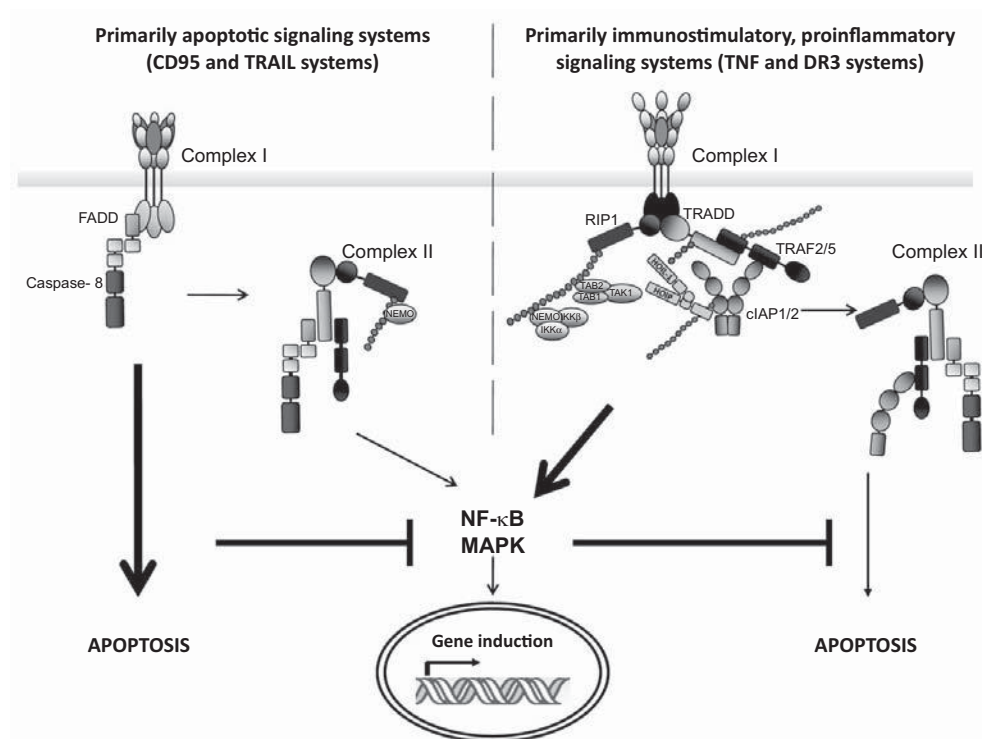


Figure 3-4. Complex I and complex II: spatial dissociation between proapoptotic and proinflammatory signaling in death receptor signal transduction. For both the CD95 and TRAIL systems, as well as the TNF and DR3 systems, the complex defined as complex I is the protein complex that forms at the plasma membrane and exerts the primary function of the respective receptor (i.e., apoptosis for CD95 and TRAIL-R1/R2 and gene induction via NF- κ B and MAPK activation by TNFR-R1 and DR3). By an undetermined mechanism, the primary adaptor protein for the different complexes I dissociates from the DD of the respective receptor, together with a number of other proteins assembled in complex I (but without the receptor), and recruits additional proteins from the cytosol. This complex II then triggers the secondary function of each receptor. In the case of proapoptotic receptors, this is gene induction via activation of NF- κ B and the MAP kinases pathways; in the case of the primarily immunostimulatory, proinflammatory receptors, it is induction of apoptosis. Successful completion of the respective primary signal interferes with the execution of the respective secondary signal. See Color Plate 6.

basis for this is. ILP is approved for unresectable soft tissue sarcoma, and it has also been successfully applied in the treatment of various other local tumors. The success of this technique proved that TNF can be used to treat cancer, albeit only when administration is locally restricted and by exerting its killing activity on an unexpected cellular target. Hence successful TNF treatment has mainly become a matter of targeted delivery to the tumor site.

A molecular way of achieving targeted delivery is to create fusion proteins in which TNF is conjugated to antibody fragments or natural ligands that specifically recognize surface proteins on tumor cells or in the tumor stroma. Using this technique, significant killing of tumor cells has been obtained, and exciting new recombinant proteins are currently being investigated. As an example, a melanoma-specific antibody conjugated to recombinant human TNF exhibited very good killing activity against TNF-resistant melanoma cells, both in

vitro and in vivo. Thus these types of fusion proteins or conjugates may not only result in more effective tumor targeting, but may also enhance the killing activity of TNE, most likely by providing membrane fixation and thereby enabling higher order receptor cross-linking on the target cell. Similar fusion proteins have also been constructed with CD95L and TRAIL. The preclinical results obtained with some of the proteins are very encouraging.

The by far most important clinical development in the TNF field to date emerged from the initially discouraging observation that TNF exerts an inflammatory response. When scientists started investigating the upside of this, they realized that interference with this response can be used to treat inflammatory conditions. In the beginning it was thought that sepsis could be targeted by inhibiting TNE. However, it was overlooked that Daniela Männel and her team, then in Heidelberg, Germany, had already shown that TNF plays an ambiguous

role in sepsis and that it is indeed required to control a sublethal sepsis, as they elegantly showed in relevant mouse models of sepsis that do not solely rely on the injection of lipopolysaccharide (LPS) (Echtenacher et al., 1995). This setback almost led to a halt of the attempts to take the inhibition of TNF forward clinically. However, when Mark Feldmann and colleagues in London, United Kingdom, then showed that inhibition of TNF interfered with collagen-induced arthritis, a model for the common human disease rheumatoid arthritis (RA), the field was suddenly revived (Williams et al., 1992). The subsequent clinical trials with RA patients returned excellent results and were successfully concluded a few years later. In the meantime, virtually millions of RA patients have benefited from therapy with TNF blockers, which have revolutionized the treatment of this disease. Other inflammatory diseases such as Crohn's disease and psoriasis, among many others, can be treated with TNF blockers, and success rates are often higher than 50%. The development of biotherapeutic TNF blockers is one of the biggest success stories in recent biomedical research. Hence the investigation of the biology of TNF receptor-ligand superfamilies has already created a huge impact on human health. Further success along these lines is eagerly awaited.

3.2. The DR3 system

On the basis of the presence of the DD and its high sequence homology with TNF-R1, in 1996 several groups discovered a third DD-containing receptor. The different groups came up with a number of different names for this receptor, but only two of these names are still used: DR3 and TNF receptor apoptosis-mediating protein (TRAMP). DR3 has been shown to bind to the TNF-like protein 1A (TL1A), an endothelial cell-derived factor. As in the case of TNF-R1, ligand-induced cross-linking of DR3 primarily activates NF- κ B and MAP kinase signaling and only secondarily induces apoptosis. The homology of the TL1A/DR3 and the TNF/TNF-R1 systems is so substantial that this system has even been referred to as the TNF system of the gut at the most recent TNF conference. This also has to do with the fact that DR3 is mainly expressed in lymphoid cells of the gut-associated lymphoid tissue (GALT), yet it is also found on cells in other lymphoid organs, including the thymus and spleen. DR3 is constitutively expressed by conventional T cells and natural killer cells. In T cells, its expression is strongly upregulated after activation.

Thus far, TL1A has only been shown to induce apoptosis after addition of cycloheximide. However, in primary T cells, TL1A has been reported to enhance

proliferation and production of IL-2 and interferon gamma induced by T-cell receptor cross-linking. The expression of TL1A is inducible in professional APCs (e.g., dendritic cells, macrophages, and B cells) and T cells, but it can also be expressed by nonimmune cells, such as smooth muscle cells and endothelial cells, during conditions of inflammation.

Less is known about DR3 signaling, but it has been suggested to interact with TRADD and FADD. However, given its high similarity with TNF-R1, it is quite likely that the early data on DR3 signaling, which were at least in part over-expression-based analyses, may have been somewhat misleading. It is quite likely that DR3 mediates gene induction in a manner similar to that of TNF-R1. However, these mechanisms are far less established for DR3 than for TNF-R1. Indeed, although DR3 was initially named death receptor 3 because of its intracellular DD, more recent functional data suggest that the activity of DR3 is mainly proinflammatory. DR3 is a strong activator of NF- κ B, and DR3 signaling can result in the increased accumulation of T cells and resistance to apoptosis. It is important to note that DR3 has been associated with the pathogenesis of RA. This is supported by the fact that DR3-deficient mice are more resistant to collagen-induced arthritis. In line with these results, activation of DR3 by TL1A exacerbated arthritis in a dose- and DR3-dependent manner. Because it was shown that treatment with TL1A-blocking antibodies protected animals from collagen-induced arthritis, targeting the DR3/TL1A pathway may represent a novel anti-inflammatory therapeutic approach for RA patients who do not respond to therapy with TNF blockers or become refractory to it. Moreover, genetic variants of TL1A and DR3 have also been associated with Crohn's disease.

Reports have also shown that DR3 is essential for other, T-cell-mediated inflammatory diseases. Using DR3-deficient mice, studies have shown that DR3 is required for TL1A-induced T-cell costimulation and that dendritic cells are the likely source of TL1A. DR3 expression on T cells has been found to be required for immunopathology, local T-cell accumulation, and cytokine production in experimental autoimmune encephalomyelitis and allergic lung inflammation, two disease models that depend on distinct effector T-cell subsets. In the development of allergic lung inflammation, which leads to asthma, DR3 is the essential trigger for IL-13 release by natural killer T cells and for amplification of the Th2 response by Th2-polarized CD4 cells. In vivo blockade of TL1A inhibits lung inflammation and production of Th2 cytokines. Accordingly, blockade of DR3 by a dominant-negative transgene

confers resistance to lung inflammation in mice (Meylan et al., 2008). Meylan et al. (2008) proposed that the interaction of TL1A with DR3 provides an early signal for Th2 cytokine production in lung inflammation and that DR3 could be a valuable target in the treatment of asthma.

4. THE DR6 SYSTEM

Although death receptor 6 (DR6) was identified already in 1998 on the basis of its similarity to TNF-R2, it is the by far least studied DD-containing receptor to date. This is most likely due to the fact that until very recently, a cellular ligand for DR6 has been elusive. In addition, unlike all other members of the DD-containing subfamily of receptors in which the DD is found at or close to the carboxy-terminus of the protein, the DD of DR6 almost immediately follows the transmembrane domain, and then there is an additional domain of approximately 150 amino acids and unknown function at the carboxy-terminus of the protein. Thus the sequence of the two intracellular portions is inversed in DR6 as compared with the other five DD-containing receptors. This difference with respect to intramolecular positioning of the DD may be the reason why DR6 seems to recruit adaptor proteins in a manner different from that of the other DD-containing receptors. DR6 has been shown to be capable of engaging a signal transduction pathway that leads to the activation of NF- κ B and JNK. However, overexpression of DR6 has been shown to induce apoptosis in a manner dependent on its DD. There are indications that this effect may be mediated via recruitment of TRADD and not FADD. However, the interaction with TRADD is a low-affinity interaction, and possibly DR6 needs a TRADD-related molecule or an additional adaptor protein to engage the cell death machinery. Taken together, it seems that the molecular interactions at the onset of DR6-induced signal transduction have largely remained in the dark, at least thus far.

Despite considerable efforts, to date no TNFSF member that binds to DR6 has been identified. However, in a recent study (Nikolaev et al., 2009), a non-TNFSF protein with an interesting etiology was described as a ligand of DR6. This protein is a specific proteolytically processed form of APP. APP is a transmembrane glycoprotein that undergoes shedding. APP is thought to be causally implicated in Alzheimer's disease (AD). Trophic factor deprivation in neurons leads to cleavage of APP by β -secretase, followed by further cleavage of the released fragment by an unknown protease, thereby generating the N-terminal 35-kDa fragment of APP (N-APP), which is capable of binding to DR6. The capacity of N-APP to bind to DR6 was identified in COS cells and by an enzyme-linked immunosorbent assay–

like binding assay. Specificity of the interaction between N-APP and DR6 has been tested in pull-down assays showing that N-APP does not interact with any of the other DD-containing receptors. Binding of N-APP to DR6 triggers a caspase-dependent limited cellular destruction process. It has been shown that after trophic factor deprivation, N-APP release triggers DR6-mediated death of the neuronal cell body, which involves activation of caspase-3, whereas, interestingly, axon degeneration is mediated by caspase-6 in a Bax-dependent manner. The mechanism of this differential control of caspase activation between cell body and axons is at present completely unclear. It will be particularly interesting to understand how caspase-6 can be activated without involvement of caspase-3 and also in the absence of any apparent activation of caspases-8 and -10. Because DR6 is widely expressed in neurons as they differentiate and enter a proapoptotic state and APP is highly expressed on axons, and because AD is marked by neuronal and axonal degeneration, the study proposed an involvement of DR6 in loss of neuronal cell mass in AD.

In summary, although this study (Nikolaev et al., 2009) partially illuminates our understanding of DR6-mediated processes, it also poses many basic questions regarding the mechanism of DR-mediated signaling behind. Thereby it exemplifies how little we still know about this thus far most elusive death receptor-ligand system, the biochemistry of its signaling pathways, and the physiologic role it may play. It will be exciting to follow the developments of this field as it may hold the key to the treatment of one of the worst neurodegenerative diseases we are faced with today. And who knows, maybe the TNF history will be repeated, and DR6 may even play a role in other neurodegenerative diseases.

5. FUNCTIONAL SPECIALIZATION BY SEQUENTIAL SIGNALING COMPLEX FORMATION IN DEATH RECEPTOR SIGNAL TRANSDUCTION

The receptor-associated signaling complexes described in the earlier sections of this chapter form at the plasma membrane. However, they are not the only signaling complexes that form in the cell when TNFSF ligands activate DD-containing TNFSF receptors. After formation of the receptor-associated protein complex, referred to as complex I, biochemical changes within the complex that are not yet understood induce loss of affinity of the adaptor proteins FADD and TRADD for their respective receptors. Together with at least some of the factors they recruited to the respective receptors, they then form a secondary, cytoplasmic signaling complex, complex II, which can recruit further proteins to the liberated DDs of FADD or TRADD, respectively. Intriguingly, in both cases

complex II is capable of inducing the very signal that was not induced by the respective complex I; that is, complex II, derived from TRADD-binding receptors, induces signals that can lead to apoptosis, and complex II of FADD-binding receptors induces gene activation, resulting in proinflammatory signaling. However, when the primary signals from the respective complex I prevails, the outcome of secondary signaling from complex II is often neutralized by the very effects triggered by the primary complex (Figure 3-4).

This new concept was first introduced for TNF-R1 in a landmark study by Micheau and Tschopp (2003). They found that signaling by this receptor involves the formation of two sequential signaling complexes, leading to activation of transcriptional programs and induction of apoptosis, respectively. The first complex that forms at the plasma membrane when TNF cross-links TNF-R1 induces biochemical reactions that ultimately result in the activation of transcriptional events, whereas the cytoplasmic complex II – although derived from complex I – is capable of inducing apoptosis, at least when signaling events induced by complex I do not impede this (Figure 3-4).

More specifically, release of TRADD from the receptor, together with the majority of the signaling proteins that it either directly or indirectly recruited to this complex, leads to the formation of complex II. Complex II then recruits FADD, presumably to the DD of TRADD, which is freed because it left the DD of the receptor behind. Then the initiator caspase-8 and -10 are recruited to FADD, and, initiated by this intracellular secondary DISC, the cell can now undergo apoptosis. However, the signaling outcome of complex II depends on the result of complex I signaling; the gene-inducing events triggered by complex I of the TRADD-binding receptors in most cases lead to an increase in the expression of cFLIP. This then interferes with activation of caspase-8 and -10 at complex II, the cytoplasmic DISC (Figure 3-4), with the result that the cell does not die. It is very likely that the same events are true for DR3 signaling; however, this has not yet been studied.

For the FADD-recruiting receptors, it has in turn been shown that on release of FADD from the TRAIL DISC (i.e., the complex I in this system), complex II recruits TRAF2, cIAP1/2, RIP, NEMO, and possibly a number of other proteins – including TRADD – required to induce the activation of NF- κ B, as well as the JNK and p38 MAP kinase pathways (Varfolomeev et al., 2005). Obviously, this pathway would only be induced in a productive manner in cells in which proper execution of the apoptotic cell death program, which is usually quite rapid, would be blocked. Thus this pathway is not the primary reaction of the cell to the stimulus provided by CD95L or

TRAIL but must be regarded as the secondary, alternative outcome of activation of the direct apoptosis inducers.

The spatial and temporal separation of different biochemical tasks into discrete signaling complexes that act in different cellular compartments (i.e., at the plasma membrane versus in the cytoplasm) and are activated sequentially in a hierarchical manner is striking and makes biological sense. In case the first signal prevails, you do not need the second one, and in fact it should probably be minimized. However, if the primary signal is not achieved, then the second, deferred signal kicks in and opens new avenues to achieve a very different, seemingly opposing outcome.

One may ask why the system does not try to achieve the same outcome in its second attempts. Perhaps it does, but in an unexpected manner. When a certain outcome of signaling is not achieved, then this means there is a problem in its execution. In such situations, biology often follows a new path to achieve the same physiologic end point. If a cell that should die does not do so, this means trouble. Therefore, the activation of proinflammatory signaling to attract other cells of the innate immune system (and, possibly later, also adaptive immune cells, which may be able to handle the situation around the cell that did not die) seems like a very sensible thing to do. With respect to the TRADD binders, if proper immunostimulatory signaling, as supervised by induction of cFLIP, cannot be achieved, then the induction of the cell death program kicks in. This cell death is apoptotic. Apoptotic cell death can either be immunogenic or nonimmunogenic. It is not clear which type of cell death is induced by TNF when the gene-inductive path does not prevail. However, our prediction would be that it is the immunogenic one and that thereby the same biological outcome (i.e., the creation of an immunostimulatory, pro-inflammatory environment) could be achieved, yet via a path very different from the originally intended apoptosis. Thus in the end it appears that cellular suicide and inflammation may be linked closer to each other than it first seemed.

6. CONCLUDING REMARKS AND OUTLOOK

Since the discovery of TNE, many truly exciting developments have characterized the research into the function of TNFR-like receptors that are capable of inducing cell death. The level to which the study of the different receptor-ligand systems has pushed our understanding of the biochemical processes that are at the heart of the induction of the specific cellular responses associated with both their physiologic and pathological consequences is astonishing. However, these studies have also made it very clear that we will

have to understand them in even more detail. This means that we have to be able to study them in a time-resolved manner, and we have to get close-up pictures of parts of the system to unravel the molecular simplicity behind the processes that today still seem so incredibly complex. By determining the biochemical interactions at the molecular, in some cases submolecular, atomic level, we will ultimately be able to unravel their mysteries and interpret them correctly.

Set apart from the beauty of solving the mysteries of molecular interactions and their connection to biology, the most important achievement of the research into the function of the receptors and ligands discussed in this chapter is the translation of knowledge on the basic biochemical mechanisms into clinical practice. Astonishing achievements in the TNF field have been made to date. However, a number of additional new avenues into clinical application are currently being followed within the TNF and TNFR superfamilies, and some of them have been touched on in this chapter. It seems we are far from having appreciated the full potential of the death receptor-ligand systems as targets for the treatment of diseases. So this fascinating and hopefully rewarding journey continues.

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4

Mitochondria and Cell Death

Gavin P. McStay and Douglas R. Green

1. INTRODUCTION

Cell death pathways use genetically encoded and derived components to transduce specific death-inducing signals into a common phenotype associated with death. These signals often include steps that impinge on mitochondria as a means of initiating or amplifying the process. Because mitochondria are organelles that contain unshared lipid and protein components, this makes them ideal targets for specific integration into cell death pathways at defined steps because of the actions of proteins present in the cytosol that target and respond to these unique components. Mitochondria are the site of the cell's major energy-generating system that produces adenosine triphosphate (ATP), used to maintain vital cellular functions. By being involved in these two conflicting processes, a cell ensures that mitochondrial energy generation and cell death are generally exclusive events. This is compatible with cellular fate ensuring shut-down of anabolic processes and favoring dismantling of cellular architecture and function, thus making death proceed in a swift manner. Here we discuss the function of mitochondria in apoptosis and necrosis and how these roles affect other aspects of mitochondrial biology.

2. MITOCHONDRIAL PHYSIOLOGY

Mitochondria are double membrane organelles that exist in all eukaryotic cells. They are termed the “powerhouse” of the cell because they provide energy in the form of ATP to ensure that essential cellular processes are maintained and cells can survive and/or proliferate in their surroundings. ATP generation occurs through the electron transport chain in the inner mitochondrial membrane (IMM). Metabolites derived from the tricarboxylic acid (TCA) cycle undergo a series of

enzymatic reactions generating reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂). Electrons from NADH are transported from complex I (NADH:quinone oxidoreductase) to coenzyme Q, to complex III (coenzyme Q:cytochrome c oxidoreductase), then finally cytochrome c to complex IV (cytochrome c oxidase), which uses these electrons to generate water from molecular oxygen. FADH₂ is generated by complex II (succinate:coenzyme Q oxidoreductase), and electrons are also transferred to coenzyme Q and follow the same pathway as NADH-derived electrons. Complexes I, III, and IV pump protons from the matrix into the intermembrane space (IMS), creating a pH and electrical gradient across the IMM (positive and acidic in the IMS, negative and basic in the matrix). This gradient is termed the mitochondrial membrane potential and is coupled to the function of many mitochondrial processes, such as protein, ion, or metabolite transport across the IMM. Regarding the generation of ATP, the IMM enzyme F₁F₀ ATP synthase drives ATP synthesis against the flow of protons from the IMS to the matrix. The IMM has a larger surface area and is folded many times within the outer mitochondrial membrane (OMM). This folding creates sub-organellar compartments known as cristae that are enriched in components of the electron transport chain, particularly cytochrome c. Mitochondria are the main source of reactive oxygen species (ROS) in a cell, mostly through the inefficient transfer of electrons between components of the electron transport chain. Therefore, mitochondria are very susceptible to the damaging effects of these highly reactive intermediates. Damage to mitochondrial proteins, lipids, and/or DNA must be efficiently repaired to maintain proper function. However, mitochondria that have received too much damage may be removed entirely through mitochondrial-specific autophagy (mitophagy)

or can fuse with less damaged counterparts, allowing damaged proteins, lipids, or DNA to be diffused or repaired. Mitochondrial fusion and fission events allow the mitochondrial network to remain a relatively homogeneous population and ensure equal division of mitochondria during cell division.

3. THE MITOCHONDRIAL PATHWAY OF APOPTOSIS

Several cell death pathways impinge on mitochondria as a means of initiating or amplifying cell death signaling. During the decision processes of apoptotic cell death, the BCL-2 family of proteins is engaged to regulate the release of mitochondrial IMS proteins, such as cytochrome c, into the cytosol in a process termed mitochondrial outer membrane permeabilization (MOMP). MOMP engages the mitochondrial pathway of apoptosis mainly by the release of cytochrome c. On its release, cytochrome c induces the activation of caspases, a family of proteases that cleave hundreds of intracellular substrates. This activity ensures that dying cells targeted by apoptosis are dismantled, recognized and removed in a manner that minimizes damage to the surrounding tissue.

4. MITOCHONDRIAL OUTER MEMBRANE PERMEABILIZATION

Mitochondria are engaged in the apoptotic pathway through the action of the BCL-2 family (described in detail elsewhere in this volume) characterized by one or more BCL-2 homology domains (BH1–4). Some proapoptotic members of this family contain only the third BH domain and are known as the BH3-only proteins; specific members of this subset include BID, BIM, PUMA, and BAD, among others. The other type of proapoptotic proteins are known as the multidomain proapoptotic effector proteins. This subset is composed of the pore-forming proteins BAX and/or BAK. The antiapoptotic BCL-2 proteins contain all four BH domains and include the proteins BCL-2, BCL-xL, and MCL-1. A subset of BH3-only proteins can directly activate BAX or BAK inducing pore formation in the OMM (Figure 4-1). Antiapoptotic proteins inhibit the activity of BH3-only proteins by binding and thus preventing activation of BAX and BAK. Other members of the BH3-only proteins can only bind to the antiapoptotic proteins and are able to displace bound direct activators that can activate BAX and BAK. Once activated, BAX and BAK are thought to form oligomeric pores that allow the release of IMS proteins, including cytochrome c, into the cytosol. The OMM acts as a platform for BAX and BAK

activation by the presence of specific components. This is demonstrated as lipid vesicles require the mitochondrial lipid, cardiolipin, or resident OMM proteins to be permeabilized by BAX, and vesicles lacking cardiolipin or resident OMM proteins resist permeabilization.

5. MORPHOLOGICAL CHANGES IN MITOCHONDRIA DURING MOMP

A frequent feature of the mitochondrial network during apoptosis is that it undergoes fragmentation, changing from a reticular network to punctate structures in the cell (Figure 4-2). The importance of this event has not been elucidated but has led to the hypothesis that proteins involved in mitochondrial dynamics participate in BAX/BAK activation and MOMP. Dynamin-related protein-1 (DRP-1) is responsible for constricting the OMM and IMM to induce mitochondrial fission. Removing DRP-1 function can inhibit or delay MOMP and apoptosis. However, the mechanism of inhibition may not be due to inhibition of mitochondrial fission but rather directly on the BCL-2 family. Evidence suggests that fused mitochondria are able to release cytochrome c after apoptotic stimuli, supporting the role of the mitochondrial fission and fusion proteins as regulators of BAX/BAK activation and not fission or fusion per se as a mediator of MOMP.

Another protein involved in mitochondrial dynamics implicated in MOMP regulation is optic atrophy-1 (OPA-1). This protein resides in the IMM and IMS as either short or long isoforms derived from differential splicing and proteolysis. OPA-1 is involved in both the fusion of IMMs and organization of cristae. Cristae contain approximately 85% of total cytochrome c in mitochondria, whereas approximately 15% is localized to the IMS directly beneath the OMM. OPA-1 exists as oligomers (possibly as a trimer made up of two IMM-bound isoforms and one IMS-soluble isoform) at the cristae openings (Figure 4-1). On BAX or BAK activation, these complexes dissociate, allowing the efflux of cytochrome c from the cristae space that is now continuous with the IMS and eventually through the BAX/BAK pore at the OMM. Inhibition of OPA-1 expression supports enhanced cytochrome c release and apoptosis, whereas OPA-1 overexpression is suggested to inhibit MOMP.

6. DOWNSTREAM OF MITOCHONDRIAL OUTER MEMBRANE PERMEABILIZATION

The actions of BAX or BAK on the OMM cause the release of many proteins from the IMS (Figure 4-2). Once MOMP has occurred, cytochrome c, which normally

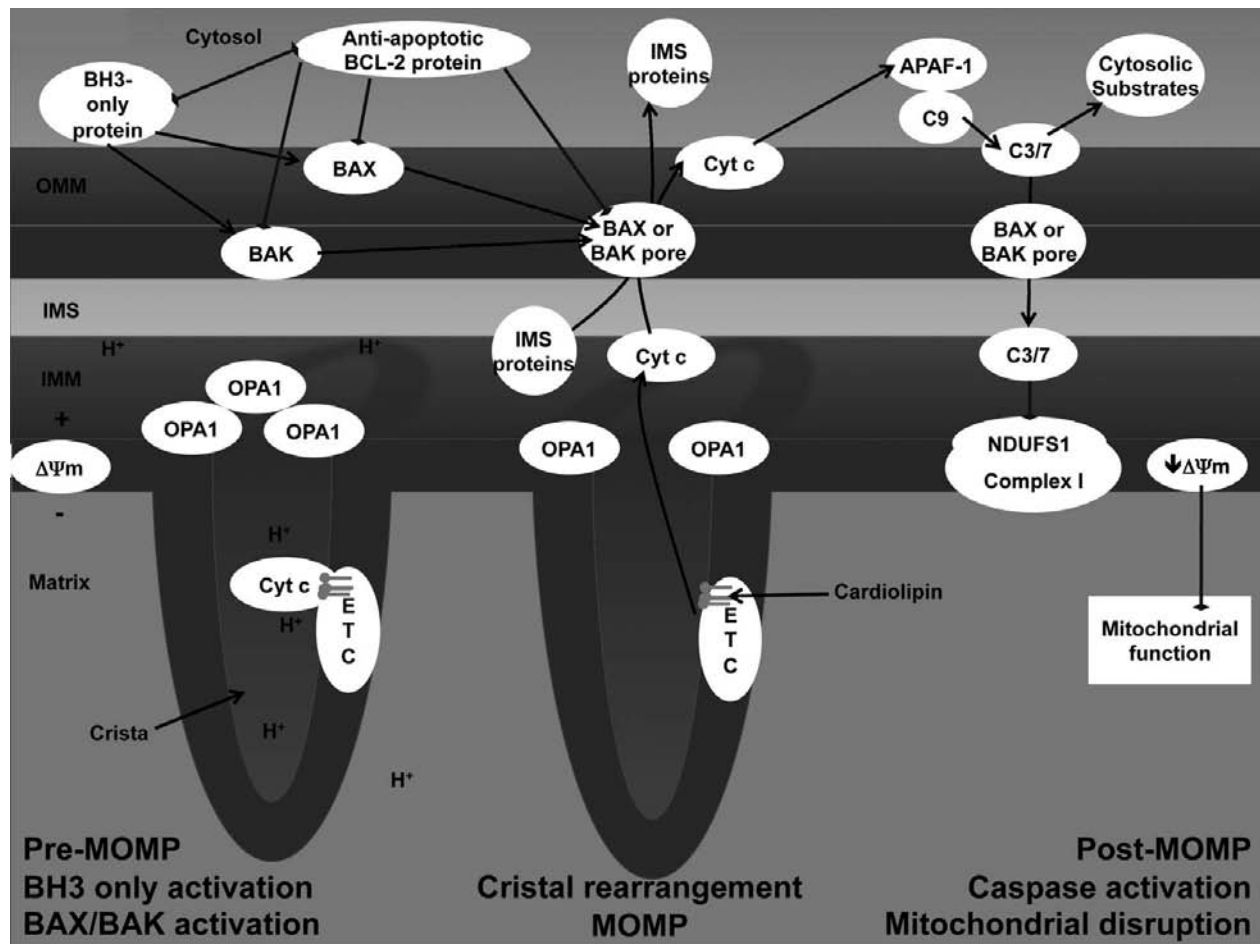


Figure 4-1. Events that occur at the inner and outer mitochondrial membranes upstream and downstream of mitochondrial outer membrane permeabilization. C9, caspase-9; C3/7, caspase-3/caspase-7; cyt c, cytochrome c; ETC, electron transport chain; IMM, inner mitochondrial membrane; IMS, intermembrane space; MOMP, mitochondrial outer membrane permeabilization; OMM, outer mitochondrial membrane. See text for full description.

resides in the IMS, is released into the cytosol, resulting in the activation of caspases. In the cytosol, cytochrome c binds to apoptotic protease activating factor-1 (APAF-1), which in concert with dATP causes a conformational change in APAF-1, which results in oligomerization into a heptameric structure called the apoptosome (Figure 4-2). The apoptosome acts as a scaffold for the recruitment and activation of procaspase-9. Dimerization of caspase-9, via the apoptosome, induces its proteolytic activity. Caspase-9 then cleaves and thereby activates caspase-3 and -7, proteases responsible for the cleavage of many cellular proteins that result in the phenotypic hallmarks of apoptosis, such as cutting of DNA into small fragments, condensation of chromatin in the nucleus, dissipation of mitochondrial membrane potential, and redistribution of phosphatidylserine (PS) from the inner leaflet to the outer leaflet of the plasma membrane.

Other proteins accompany cytochrome c during MOMP. These include SMAC/Diablo (second mitochondrial activator of apoptosis/direct IAP binding protein with low pI) and Omi/HtrA2, both of which assist in caspase activation by antagonizing the inhibitor of apoptosis proteins (IAPs), a family of proteins that inhibit caspases directly. Activated caspases-3, -7 and -9 are potentially inhibited by X-linked inhibitor of apoptosis protein (XIAP), but this inhibition can be relieved by the action of IAP antagonists, especially SMAC/Diablo through its IAP-binding motif (IBM) that disrupts IAP:caspase complexes. Therefore, the main role of SMAC/Diablo may be ensuring a concerted burst of caspase activity downstream of MOMP. Omi/HtrA2 also contains an IBM and may inhibit IAP function through a similar mechanism.

There are two other proteins that may play important roles in cell death on release from mitochondria;

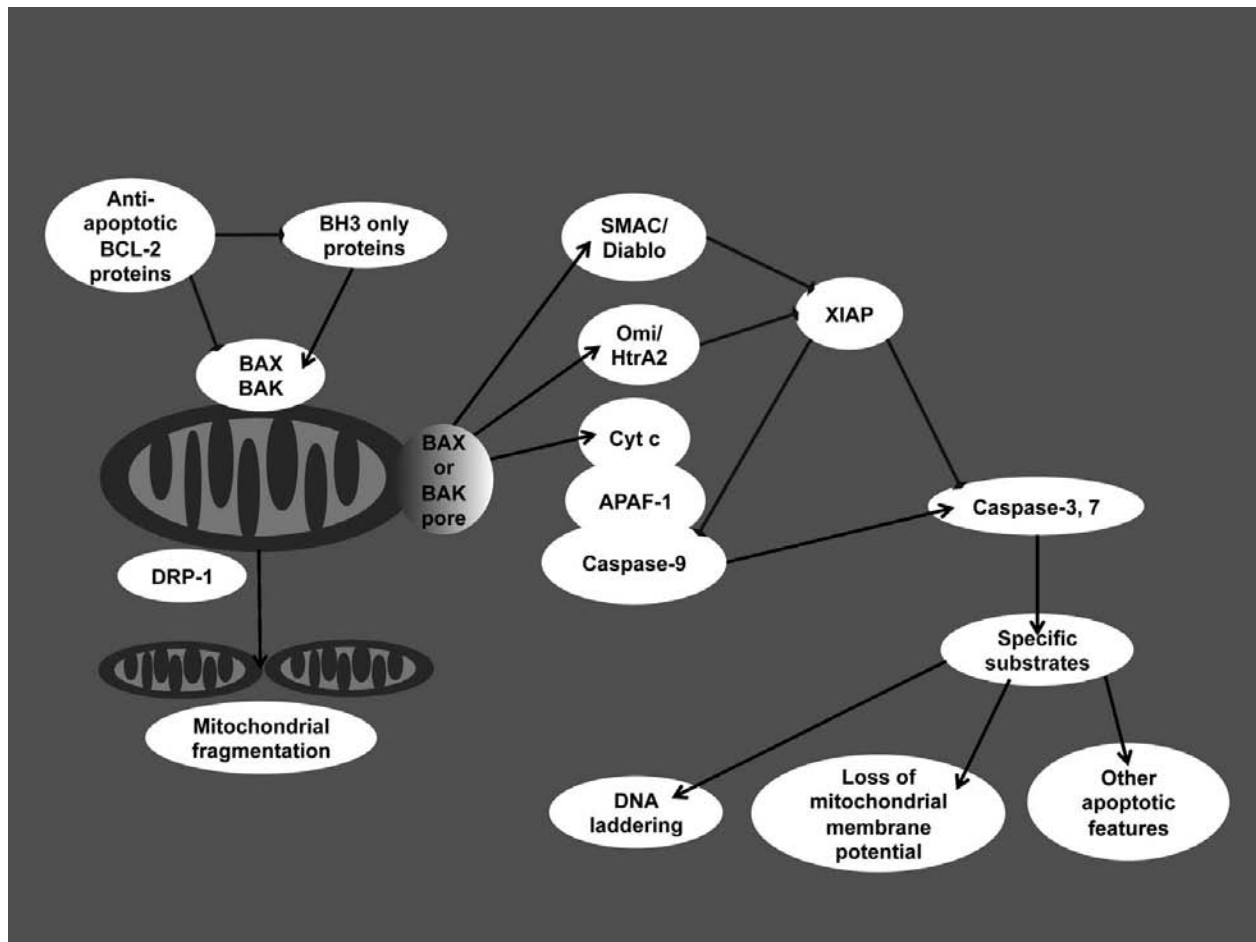


Figure 4-2. Events that occur downstream of mitochondrial outer membrane permeabilization with focus on proteins released from the intermembrane space and their effects on cytosolic components. cyt c, cytochrome c; XIAP, X-linked inhibitor of apoptosis protein. See text for full description.

however, their mechanisms of release are unclear. Apoptosis-inducing factor (AIF) exists in the IMM and appears to play a role in mitochondrial complex I assembly or function. Once MOMP has occurred, AIF can translocate from mitochondria into the cytosol, but this event may depend on caspases and/or calpains, placing AIF as a mediator of cellular dismantling rather than as an initiator of cell death. AIF has also been suggested to act as a direct mediator of DNA fragmentation. Lastly, endonuclease G, which is present in the mitochondrial matrix, is reportedly released after MOMP, and this protein might also cause DNA fragmentation independently of caspases. Other proteins have been described that are released during MOMP (e.g., adenylate kinase-2), but these may be only bystanders with no specific proapoptotic function.

Disruption of the OMM dilutes cytochrome c, but sufficient protein can be present to sustain electron transport. However, activated caspases gain access to the IMS and can specifically disrupt mitochondrial function.

Oxidative phosphorylation activated by complexes I and II of the electron transport chain are inhibited when caspases are activated. Caspase-3 cleaves and inactivates complex I at a specific site in the subunit NDUF51 (NADH dehydrogenase [ubiquinone] Fe [Iron]-sulfur 1) (Figure 4-1). This inhibits complex I activity and disrupts electron transport and ultimately mitochondrial function through dissipation of the mitochondrial membrane potential. Preventing the cleavage of NDUF51 subunit delays apoptosis and delays PS exposure on the plasma membrane, perhaps through maintaining ATP levels in the cell. Also, mitochondria that have undergone MOMP are targets for mitophagy. This removes the damaged organelles from cells and its detrimental effects such as ROS generation or excessive substrate (such as ATP) consumption.

Even though MOMP usually leads to caspase activation, most cells die, even if caspase activity is defective. In situations in which caspase inhibitors are present or caspase-activating proteins (e.g., APAF-1) are absent,

cells submit to caspase-independent cell death (CICD). Cells die in this process as a result of the loss of energy-producing capability of mitochondria as the mitochondrial membrane potential slowly dissipates because of impaired oxidative phosphorylation. To maintain mitochondrial membrane potential, ATP is hydrolyzed by F_1F_0 ATP synthase, but eventually ATP is depleted, and this leads to a slow, uncontrolled, necrotic death. This process can be inhibited by over-expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a glycolytic enzyme that is able to sustain ATP concentrations through glycolysis and also engage autophagy to remove mitochondria that have undergone MOMP. Cells undergoing CICD are able to proliferate after MOMP when caspase activation is inhibited and GAPDH is over-expressed, suggesting that sustaining energy production and removal of damaged mitochondria is enough to ensure cell survival after MOMP.

7. MITOCHONDRIAL PERMEABILITY TRANSITION PORE AND NECROTIC CELL DEATH

As mitochondria are responsible for energy production they are able to sense the metabolic status of a cell. In pathological situations such as ischemia-reperfusion, when blood flow is temporarily restricted followed by return to normal blood flow, or other prolonged cellular stresses when metabolic substrates become limiting or metabolic end-products accumulate, mitochondria respond by opening the mitochondrial permeability transition pore (MPTP). This pore forms in the IMM and is composed, in part, of cyclophilin D (CyP-D) in the matrix. Other suggested components include the adenine nucleotide translocase (ANT), but this is controversial (Figure 4-3). The opening of the MPTP allows exchange of mitochondrial matrix and cytosolic components of less than 1,500 D, destroying the compartmentalization of the mitochondria, as evidenced by the dissipation of the mitochondrial membrane potential. Loss of the mitochondrial membrane potential prevents most functions of mitochondria. In the absence of a membrane potential, mitochondria in a cell are no longer able to produce ATP using oxidative phosphorylation and will eventually die through necrosis when critical functions are lost because of the decrease of ATP levels.

The composition of the MPTP is still unresolved, but many candidates have been proposed. The most convincing component of the MPTP is the matrix peptidyl-prolyl *cis-trans* isomerase cyclophilin D (CyP-D). In mice that lack this protein, MPTP formation is virtually undetectable, and inhibitors of this enzyme prevent MPTP. Cells derived from CyP-D-deficient mice show

inhibition of death in situations of necrosis induced by hydrogen peroxide or ischemia reperfusion in isolated hearts. However, cells from these mice do not show any resistance to apoptotic stimuli, demonstrating that the MPTP is not involved in releasing cytochrome c during apoptosis.

CyP-D is not thought to be the pore-forming unit of the MPTP, but rather an initiator of a conformational change in an IMM protein. Most speculation has focused on the adenine nucleotide translocase (ANT), the mediator of ADP/ATP exchange between the mitochondrial matrix and cytoplasm. This is mainly due to the number of known ligands of the ANT that influence MPTP opening. However, mitochondria from mice lacking all isoforms of this protein can still undergo MPTP opening, leading to the suggestion that another component may be the pore-forming unit (e.g., the phosphate carrier). Alternatively, it is possible that many similar proteins may be able to form the MPTP, especially members of the mitochondrial carrier family, of which the preceding two candidates are members. This has also led to the hypothesis that the pore is not formed by a specific protein but is due to misfolding of IMM proteins that are induced by the stresses known to engage the MPTP. In addition, OMM components may be able to influence the opening of the MPTP, such as VDAC, the peripheral benzodiazepine receptor, and creatine kinase, mainly based on experiments using specific ligands of these components or through the isolation of proteins complexes that contain putative components of the MPTP. Their roles in the MPTP remain controversial.

The MPTP forms in the IMM under certain conditions mainly driven by accumulation of excess calcium ions in the mitochondrial matrix and can be sensitized by metabolic changes within mitochondria, such as oxidative stress, adenine nucleotide depletion, high mitochondrial matrix pH, or low mitochondrial membrane potential. Insults such as ischemia-reperfusion in the heart or brain result in a large wave of necrotic cell death that is initiated by metabolic stress within the affected organ, such as bursts of reactive oxygen species and depletion of metabolites, known activators of MPTP opening.

8. COMPARISON OF THE VERTEBRATE AND INVERTEBRATE PATHWAYS OF MITOCHONDRIAL CELL DEATH

The mitochondrial pathway of apoptosis, described previously, is prevalent in vertebrate organisms; however, other pathways with homologous proteins exist in invertebrates. Differences exist between two model organisms, the nematode (*Caenorhabditis elegans*) and the

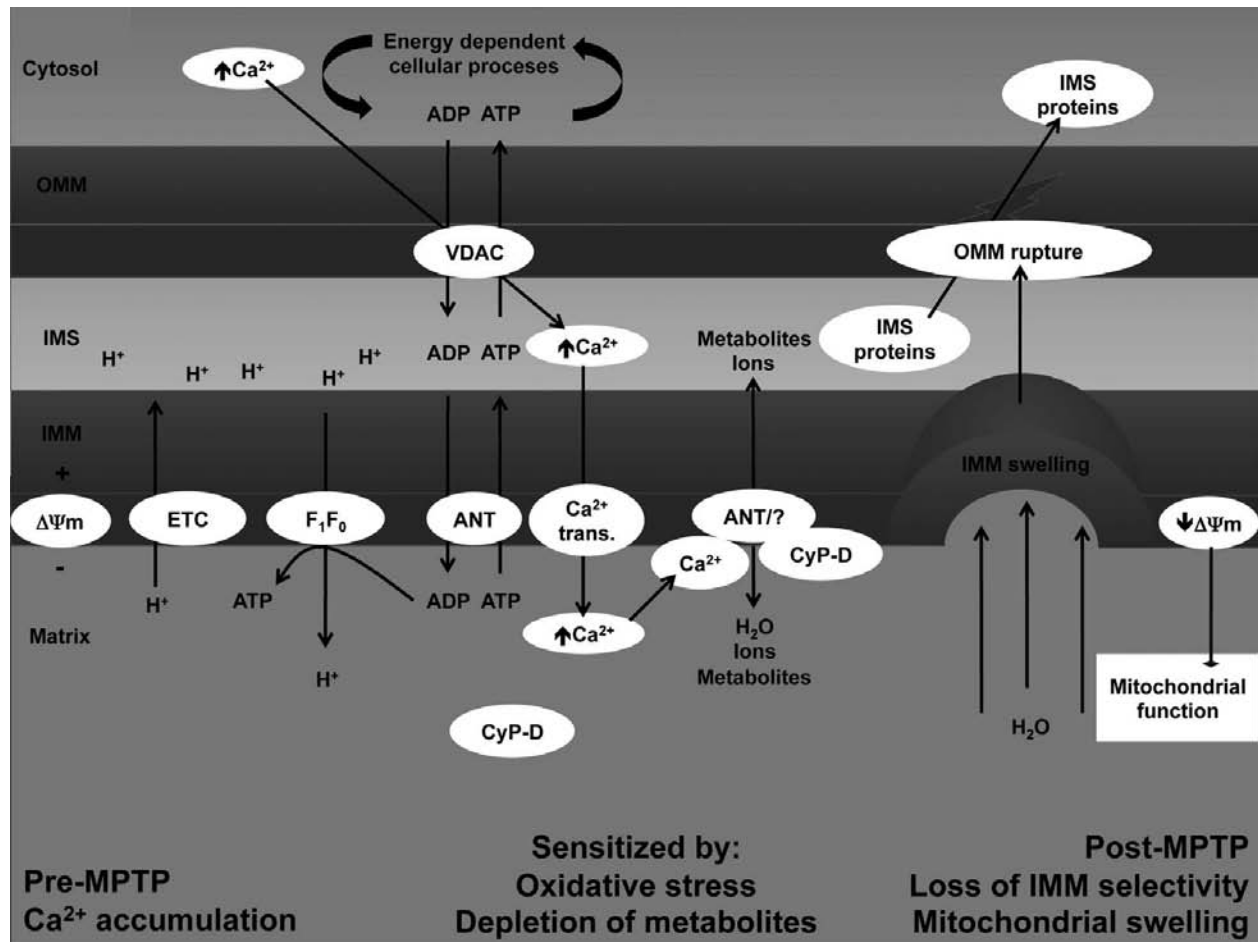


Figure 4-3. Mitochondrial changes associated with mitochondrial permeability transition pore opening. ADP/ATP, adenosine di/tri-phosphate; ANT, adenine nucleotide translocase; Ca^{2+} trans., Ca^{2+} uniporter; CyP-D, cyclophilin-D; ETC, electron transport chain; F_1F_0 , F_1F_0 ATP synthase; IMM, inner mitochondrial membrane; IMS, intermembrane space; MPTP, mitochondrial permeability transition pore; OMM, outer mitochondrial membrane; VDAC, voltage-dependent anion channel. See text for full description.

fruit fly (*Drosophila melanogaster*). In *C. elegans*, apoptosis occurs by a pathway that does not rely on MOMP, but mitochondria appear to play a role. In this pathway, the antiapoptotic BCL-2 homolog cell death abnormality (CED)-9 exists in a complex with CED-4 (the APAF-1 homolog) at the OMM. This complex is disrupted by the BH3-only homolog EGL-1, allowing activation of CED-4 and ensuing activation of the caspase-3 homolog CED-3. CED-9 has also been shown to regulate mitochondrial fission and fusion, similar to the effects of the mammalian BCL-2 family on mitochondria. Another similarity to mammalian apoptosis is the fragmentation of mitochondria during apoptosis in *C. elegans*. This is due to EGL-1-mediated binding to CED-9 and regulation of the *C. elegans* DRP-1 or mitofusin-2 homologs. Moreover, enhanced expression of DRP-1 has been suggested to induce mitochondrial fragmentation and cell death. Ectopic expression of CED-9 in mammalian cells

is able to induce mitochondrial fusion, but is unable to inhibit release of IMS proteins after induction of apoptosis, indicating that regulation of mitochondrial fission and fusion by the BCL-2 family is conserved between *C. elegans* and vertebrates.

The same holds true for apoptosis in *D. melanogaster*, where mitochondrial fragmentation has been observed. As in *C. elegans*, release of proteins from the IMS in *D. melanogaster* is not an essential process that is required for apoptosis. Although cytochrome c release has been observed during apoptosis in certain cell types in drosophila, this is a caspase-dependent event, indicating that it is a consequence of apoptosis and not an inducing event. However, the *Drosophila* homolog of DRP-1 has been shown to be involved in mitochondrial fragmentation during apoptosis. Similar to *C. elegans*, mitochondria in *Drosophila* act as a platform for apoptosis activation events as the proapoptotic IAP

antagonists Reaper, Hid, and Grim (RHG) localize to mitochondria upon their expression through a Grim homology (GH)-3 domain that binds to lipids. The primary function of RHG proteins is to antagonize the action of the *Drosophila* IAP, DIAP-1. Their action releases active caspases from DIAP-1, which then cleave important proapoptotic substrates. How or why the OMM may be involved in this process remains unclear. In contrast to *C. elegans* and the vertebrates, BCL-2 family proteins in *Drosophila* do not affect cell survival or OMM integrity.

9. CONCLUSIONS

Mitochondria play a dual role in the fate of a cell: mitochondria provide the majority of energy in the form of ATP, yet mitochondria translate cell death signals initiated by other cells but also from within themselves. In the mitochondrial pathways of cell death, the bioenergetic functions of mitochondria are compromised in specific ways. During apoptosis, fragmentation, loss of OMM integrity, dilution of IMS proteins, activation of caspases, and loss of mitochondrial membrane potential all contribute to the demise of mitochondrial function. On the other hand, during necrosis the IMM loses selectivity and mitochondria lose membrane potential and swell, causing OMM rupture. In addition, the death-promoting signals emanating from the mitochondria such as cytochrome c and SMAC/Diablo release coincide with or happen slightly before mitochondrial dysfunction. By specifically disrupting mitochondrial function, the cell's fate to die is favored due to the demise of cellular ATP levels and thereby loss of metabolic control coupled with positive death signals at the same time. These two phenomena make the mitochondrial steps of cell death pathways a point of no return from which it is difficult for a cell to recover. In long-lived cells, such as neurons, or cells attempting to avoid death, such as

tumor cells, mechanisms may be in place to delay cell death processes after the mitochondrial steps and allow cellular survival. By studying the role of mitochondria in cell death pathways, we have gleaned important information regarding cell death, but also insights into mitochondrial physiology.

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5

The Control of Mitochondrial Apoptosis by the BCL-2 Family

Anthony Letai

1. INTRODUCTION

A fundamental step in the commitment to apoptosis via the intrinsic pathway is mitochondrial outer membrane permeabilization (MOMP). This step allows the release of proapoptotic proteins from the mitochondrial intermembrane space into the cytoplasm. Once in the cytoplasm, they induce caspase activation, oligonucleosomal DNA cleavage, and other hallmarks of apoptosis. The details of these important events that occur downstream of MOMP are described in [Chapter 4](#). Here we restrict our attention to the molecular mechanisms by which the cell controls this critical event, molecular mechanisms that primarily involve interactions among the family of proteins known as the BCL-2 family.

The BCL-2 family gets its name from the B-cell leukemia/lymphoma-2 gene that was cloned from the breakpoint of the t(14;18) present in nearly all cases of follicular lymphoma, as well as a minority of diffuse large B-cell lymphomas. Although previously discovered oncogenes had been found to increase the rate of proliferation of malignant cells, BCL-2 was found instead to foster accumulation of malignant cells by inhibiting cell death. It was found that BCL-2 had homologs in virtually all metazoans studied, and it was functionally interchangeable with the Ced9 gene of the roundworm *Caenorhabditis elegans*. These results suggested that control of cell death by homologs of BCL-2 is a phylogenetically ancient property of metazoan cells.

In the years since the cloning of BCL-2 in 1985, an entire family of proteins has been discovered that is related to BCL-2 by sequence homology, as well as by involvement in control of apoptosis ([Figure 5-1](#)). The BCL-2 family contains proteins that induce as well as those that prevent MOMP and apoptosis. Next we describe how BCL-2 family proteins interact to adjudicate

whether the cell takes the critical step of commitment to apoptosis.

2. ACTIVATING APOPTOSIS: BAX AND BAK AND THE ACTIVATOR BH3-ONLY PROTEINS

BAX and BAK are essential effectors of apoptotic signaling at the mitochondrion. Models of combined deletion of BAX and BAK show that in the absence of BAX and BAK, MOMP cannot take place. In response to upstream death signaling, carried at least in part by select proapoptotic BH3-only proteins (see below, [Sections 4 and 5](#)), BAX and BAK are activated, homo-oligomerize, and form pores in the mitochondrial outer membrane (MOM). Although BAX and BAK are necessary components of the apoptotic pore in the MOM, it remains possible that they cooperate with other proteins to form this pore in vivo. Nonetheless, BAX alone can form pores in liposomal vesicles of sufficient size to permit efflux of cytochrome c, an intermembrane protein that is released after MOMP to assist in caspase activation by the apoptosome.

It is obvious that the mitochondrial membrane permeabilizing function of BAX and BAK must be tightly controlled. This control is not exerted primarily at the level of protein expression, as apoptosis can be observed over very short time scales, on the order of minutes, a time insufficient for alterations in BAX or BAK levels. Furthermore, BAX and BAK protein levels typically do not change significantly during execution of an apoptotic signal. How then are BAX and BAK converted from quiescent proteins compatible with survival to rapid and essential effectors of cell death?

The main steps for BAX activation are translocation to the mitochondrion, conformation change, insertion into the mitochondrial membrane, oligomerization,

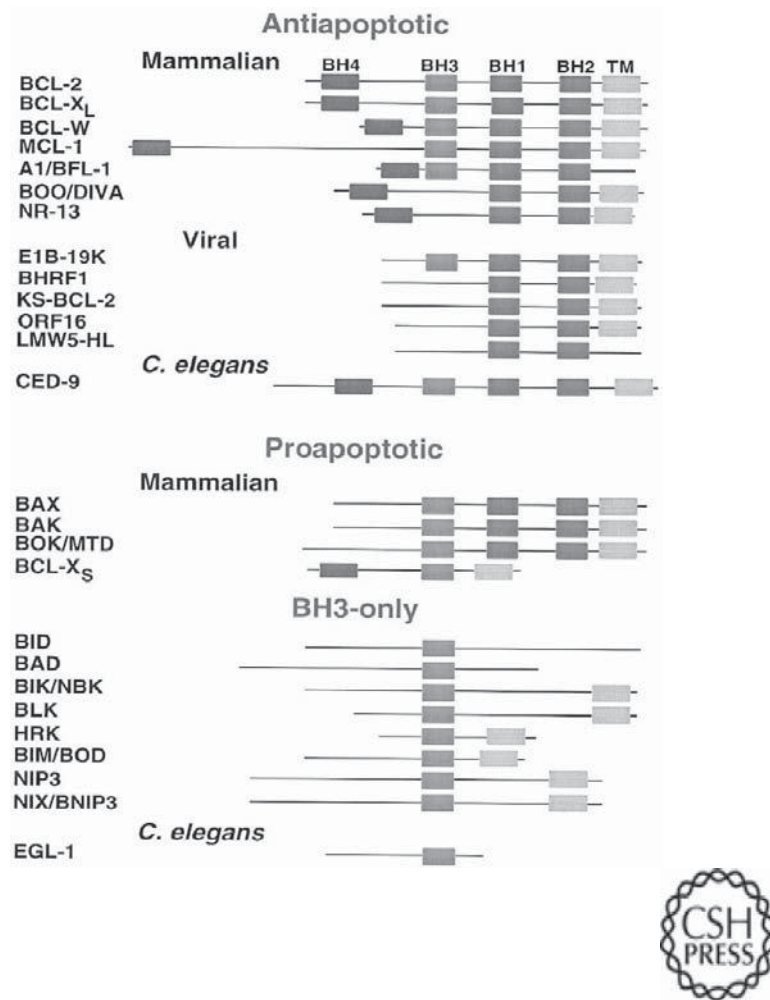


Figure 5-1. Summary of anti-apoptotic and pro-apoptotic BCL-2 members. BCL-2 homology regions (BH1-4) are denoted as is the carboxy-terminal hydrophobic (TM) domain. Reprinted with permission from Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev.* 1999;13:1899–911. Copyright © 1999 by Cold Spring Harbor Laboratory Press.

and, finally, pore formation. These events can take place over mere minutes in a cell enduring death signaling. In the case of BAX, subcellular localization is an important mode of control. Before the induction of apoptosis, BAX exists as a monomer either in the cytosol or loosely attached to the mitochondrial outer membrane in an alkali-labile fashion. Control over translocation from the cytosol to the mitochondria is poorly understood, but enforced dimerization of BAX has been observed to cause translocation, as does cytosol from cells undergoing apoptosis and the protein Peg3/Pw1. BCL-2 and Ku70 have been found to inhibit translocation of BAX to mitochondria. Because the distribution of BAX between a purely cytosolic state and one in which it is loosely attached to the mitochondria varies considerably among cell types, it seems likely that control over this localization may vary similarly.

Once localized to the mitochondrion, BAX must undergo a conformational change and insert into the mitochondrion to execute its mitochondrial permeabilizing function. Because BAK is already inserted into the mitochondrion, it requires only conformational change, oligomerization, and pore formation for complete activation. In both cases, the conformational change exposes an N-terminal epitope that can be specifically recognized by conformation-specific antibodies. This insertion and conformational change can be induced by a subset of BH3-only proteins called activators, which includes BID and BIM, and possibly PUMA (see also discussion that follows). The interaction between activators and BAX and BAK appears to be a catalytic “hit and run” type interaction, and complexes between activators and BAX and BAK are difficult, though not impossible, to observe. The importance of activators may perhaps best be seen in defined liposomal systems. In these systems, unilamellar liposomal vesicles are made with phospholipids that mimic mitochondrial composition. Recombinant BCL-2 family proteins can be added to the vesicles, and the ability to permeabilize can be measured. BAX or BID by themselves are unable to efficiently permeabilize the liposomes, but if BID is added to BAX,

permeabilization becomes drastically more efficient. Furthermore, BAX is recruited and inserted into the membrane, and a conformational change exposing its N-terminus is induced. Stoichiometric studies show that BID can activate fully a great molar excess of BAX, supporting the model of a transient “hit and run” interaction. Thus all the important properties of activation of BAX can be recapitulated by the addition of BID protein. Other proteins that have been implicated as activators of BAX and BAK include the BH3-only protein PUMA and p53, and it is possible that other molecules, perhaps even non-proteins, can participate as activators. In addition, increasing temperature to 43°C has also been found to activate BAX and BAK, likely either by alteration of the proteins themselves or perhaps alteration of the lipid environment.

Once activated, BAX and BAK oligomerize. Predominantly homo-oligomers have been observed, although it may be that oligomerization of one facilitates the oligomerization of the other. After oligomerization, BAX and BAK participate in the formation of a pore that permits the efflux of macromolecules from the intermembrane space. It is not known whether additional proteins are required for this permeabilization. Supporting an independent role for BAX and BAK is the finding that BAX can, by itself, create pores that permit the efflux of cytochrome c in liposomes. Furthermore, conductance studies suggest that the properties of channels isolated from cells and those made from recombinant BAX are similar. However, it cannot be ruled out that other proteins participate. It appears that this permeabilization is independent of the mitochondrial permeability transition pore (PTP) on the basis of the generally observed insensitivity of MOMP to cyclosporine A treatment and to deletion of cyclophilin D, a key component of the PTP. Another important channel in mitochondria is the voltage-dependent anion channel (VDAC). MOMP appears independent of this channel as well, as deletion of VDAC proteins again has little effect on MOMP.

BOK is a proapoptotic BCL-2 family protein that by sequence homology and function resembles BAX and BAK. Little is known about this protein, although its expression seems to be largely limited to reproductive tissues. Because loss of BAX and BAK alone appears sufficient to prevent MOMP in several cell types, it appears that BOK does not play an important role in many tissues. However, study of this protein is quite limited, at least in part by the paucity of good antibodies that selectively recognize BOK. It remains possible that BOK is an important effector of apoptotic signaling in response to select stresses in certain tissues.

3. INHIBITING APOPTOSIS

As might be expected with such an important cell fate decision, there are additional factors that negatively regulate commitment to apoptosis (Figure 5-2). Most prominent among these are the antiapoptotic BCL-2 family proteins, the best studied of which include BCL-2, BCL-X_L, MCL-1, BCL-w, and BFL-1. As mentioned previously, BCL-2 was the first of these proteins to be discovered and thus lends its name to the entire protein family. Forced expression of any of these antiapoptotic proteins allows a cell to survive a wide variety of insults that might otherwise induce apoptosis, including growth factor withdrawal, DNA damage, microtubule disruption, and kinase inhibition. These proteins inhibit

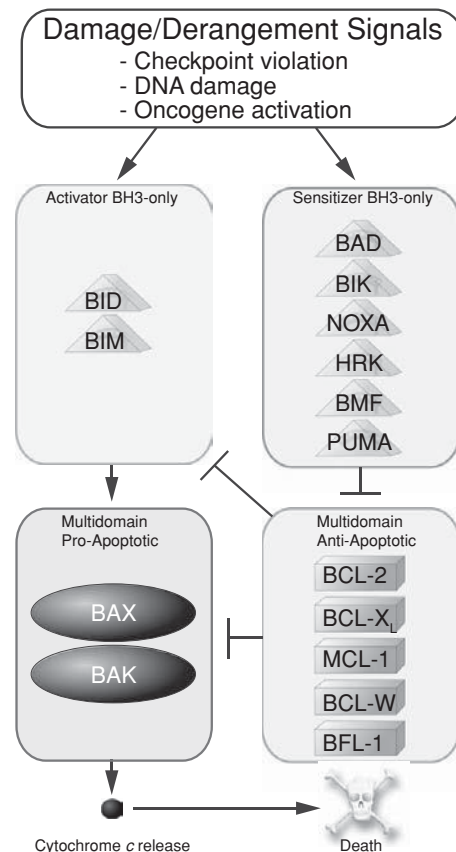


Figure 5-2. Model of BCL-2 family control of programmed cell death. Death signals cause induction or post-translational activation of BH3-only proteins. Activator BH3-only proteins, including BID and BIM, induce oligomerization of BAX and/or BAK, causing MOMP, cytochrome c release, and caspase activation, resulting in cell death. Antiapoptotic proteins prevent apoptosis by sequestering activator BH3-only proteins and activated BAX/BAK, upstream of BAX/BAK oligomerization. Sensitizer BH3-only proteins promote cell death by binding the antiapoptotic proteins, displacing activator BH3-only proteins to trigger BAX/BAK oligomerization. Reprinted from Certo M, Del Gaizo Moore V, Nishino M, et al. Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell*. 2006;9:351–65, Copyright © 2006 with permission from Elsevier.

cell death by binding proapoptotic proteins to inhibit the processes described previously. Perhaps their most important function is to bind and sequester activator BH3-only proteins to prevent their interaction with and activation of BAX and BAK. In fact, all of the antiapoptotic proteins identified can bind BID and BIM, as well as PUMA. Antiapoptotic proteins contain a hydrophobic pocket formed by the hydrophobic faces of the BH1, BH2, and BH3 domains. The BH3 domain of the activator proteins is itself an amphipathic helix, the hydrophobic face of which binds into the hydrophobic pocket of the antiapoptotic proteins.

In addition, antiapoptotic proteins can bind BAX and BAK, particularly after BAX and BAK undergo the conformational change that accompanies activation.

The relative importance of the activator-binding and effector-binding functions of antiapoptotic proteins is not completely established. It may well be that the importance varies with type of cell or insult. It is worth making an important technical note in the quantitation of complexes between antiapoptotic proteins and BAX and BAK. The conformational change in BAX and BAK can be elicited simply by exposure to nonionic detergents commonly used in the preparation of cell lysates such as Triton X-100 or NP-40. Other detergents, such as 3-[3-cholamidopropyl-dimethylammonio]-1-propane-sulfonate (CHAPS), lack this property. This induction of conformational change is furthermore associated with an increased binding of BAX and BAK to antiapoptotic proteins like BCL-2. Therefore, incautious use of detergents can result in a very significant artifactual overestimation of complexes between antiapoptotic proteins and BAX and BAK. When detergents like CHAPS are used to make whole-cell lysates, only a small minority of BAX and BAK appear to be sequestered by antiapoptotic proteins, suggesting that factors distinct from antiapoptotic protein binding are critical in controlling BAX and BAK activation.

Other proteins have been suggested to inhibit BAX or BAK activation. Humanin, a small but interesting polypeptide for which an open reading frame is present in both the nuclear and mitochondrial genomes, has been found to inhibit activation of BAX by BID by binding BID. VDAC2 has been shown to bind to BAK and negatively regulate its activation.

4. INHIBITING THE INHIBITORS

An additional layer of modulation exists for the BCL-2 family control of MOMP, composed of the sensitizer BH3-only proteins. These proteins all possess a BH3 domain, but they lack the ability to activate BAX or BAK and thus are classified as sensitizers. Unlike activators, sensitizers demonstrate a more selective pattern of interaction with antiapoptotic proteins (Table 5-1). For instance, the sensitizer BH3-only protein BAD binds to BCL-2, BCL-XL, and BCL-w, but not to BFL-1 or MCL-1. In contrast, NOXA binds to MCL-1 but not to BCL-2, BCL-XL, or BCL-w. The sensitizers are all pro-death proteins, but they exert their effect by being inhibitors of the antiapoptotic proteins. If the antiapoptotic protein is previously unbound by activators, then the interaction with a sensitizer serves to simply neutralize its function and decrease the remaining antiapoptotic reserve. If, alternatively, the antiapoptotic protein is already bound by an activator, then the binding of a sensitizer will displace the activator, allowing it to activate BAX or BAK.

It can be seen, therefore, that the commitment to apoptosis depends on a balance of pro- and antiapoptotic proteins, but one significantly more complex than originally described in a simple rheostat model, when only BAX and BCL-2 were participants. The interactions described previously are summarized in Figure 5-2.

5. ACTIVATING THE ACTIVATORS – CONNECTING THE INSULT TO THE BCL-2 FAMILY

There are many treatments that are known to commit cells to the fate of apoptosis. Yet very often, the details of how the initiating insults communicate a death signal to the BCL-2 family are poorly understood. Examples follow in which some of the important steps have been identified. These examples demonstrate the wide variety of mechanisms that are employed in provoking cell death by apoptosis.

In response to many types of DNA damage, p53 drives a response that results in either senescence or apoptosis. The apoptosis response ultimately uses the intrinsic, mitochondrial apoptotic pathway, resulting in MOMP. Much of the p53-generated signaling is due to the transcriptional upregulation of the proapoptotic BH3-only protein, PUMA. Whether PUMA acts primarily as an activator or a sensitizer is a matter of debate. However, in its sensitizer role, PUMA is particularly potent because it can bind to and neutralize all of the identified antiapoptotic proteins. In certain models of DNA damage, loss of PUMA alone can nearly phenocopy loss of p53. Transcriptional induction of other proapoptotic proteins, including NOXA and BAX, also contribute to the apoptotic response.

Intriguingly, there is some evidence that p53 can induce apoptosis independent of any modulation of transcription. Some have found that in response to DNA damage, p53 can migrate to the mitochondrion and act as both a sensitizer and an activator to directly induce MOMP. It is notable that p53 lacks a discernible BH3 domain, so that this interaction with BCL-2 family proteins is likely different from that involving BH3 domains.

In many cells, particularly those designated as type II cells, incorporation of the mitochondrial apoptotic pathway is necessary for induction of apoptosis downstream of ligation of the tumor necrosis factor (TNF) family of receptors. The key connector of the extrinsic and intrinsic pathways in this situation is the activator BH3-only protein BID. In response to ligation of their extracellular domains, receptors that include CD95/FAS, TNF, and TNF-related apoptosis-inducing ligand (TRAIL) assemble a death-inducing signaling complex (DISC). A component, the protein FADD/MORT, recruits

Table 5-1. Selective binding between antiapoptotic and BH3-only family members

	BID	BIM	BIDmut	BAD	BIK	NOXAA	NOXAB	HRK	BNIP	PUMA	BMP
BCL-2	66(6)	<10	–	11(3)	151(2)	–	–	–	–	18(1)	24(1)
BCL-XL	12(9)	<10	–	<10	10(2)	–	–	92(11)	–	<10	<10
BCL-w	<10	38(7)	–	60(19)	17(12)	–	–	–	–	25 (12)	11(3)
MCL-1	<10	<10	–	–	109(33)	19(2)	28(3)	–	–	<10	23(2)
BFL-1	53(3)	73(3)	–	–	–	–	–	–	–	59(11)	–

Note: Dissociation constants for interactions between antiapoptotic BCL-2 family proteins (left) and BH3 domains from BH3-only proteins (top) are shown in nM. Standard deviations of at least three independent measurements are in parentheses. Minus sign signify no observed binding ($K_d > 2500$ nM). BID and BIM are activators, the remainder are sensitizers.

Source: Adapted from Certo M, Del Gaizo Moore V, Nishino M, et al. Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell*. 2006;9:351–65, Copyright © 2006 with permission from Elsevier.

procaspase-8 to the complex. Perhaps via an “induced proximity” mechanism, procaspase-8 is then auto-proteolytically cleaved into its active caspase-8 form. Caspase-8 can then cleave and activate effector caspases like caspase-3 and -7. In type I cells, such as thymocytes, no further involvement of the mitochondrial pathway is necessary to commit the cell to apoptosis, and BCL-2 cannot inhibit apoptosis. However, in type II cells, BCL-2 can inhibit death, and the mitochondrial amplification loop is required for apoptosis. Cleavage of BID into the 15-kDa truncated BID (tBID) can efficiently induce activation of BAX and BAK and cause MOMP. The activator function of tBID can be further enhanced by enzymatic myristoylation of the glycine residue on the new amino-terminus of the tBID molecule, as it facilitates targeting to the mitochondrion.

Although BH3-only proteins are usually the most dynamic players in the BCL-2 family in response to noxious stimuli, MCL-1 levels can also dramatically change in response to select death signaling events. A prominent example is growth factor withdrawal. When interleukin (IL)-3 is removed from the culture of IL-3-dependent cell lines, including FL5.12, 32D, and Ba/F3, there is a reduction in PI3K activity. This results in decreased activated AKT, resulting in a release of increased glycogen synthase kinase 3 (GSK-3) activity. GSK-3 phosphorylates MCL-1, targeting it for ubiquitinylation and proteasomal degradation. The shortening of the already short half-life (on the order of minutes) of MCL-1 results in a dramatic lowering of MCL-1 levels, freeing BIM that had been sequestered by MCL-1 to activate BAX and BAK and commit the cell to apoptosis. In contrast, in an IL-6-dependent model, withdrawal of IL-6 yielded a decrease in MCL-1 levels as a result of decreased transcription of MCL-1.

Subcellular localization can also play an important role in modulating function of BCL-2 family proteins. For

instance, in its phosphorylated state, the proapoptotic sensitizer BH3-only protein BAD is sequestered by 14-3-3 in the cytoplasm. In IL-3-dependent cells, presence of IL-3 induced BAD phosphorylation. When dephosphorylated, BAD migrates to the mitochondrion to bind to antiapoptotic proteins such as BCL-2 and BCL-XL to promote death. It has been suggested that the BH3-only proteins BIM and BMF promote death when displaced from their cytoskeletal locations on the microtubule dynein motor complex and actin-based myosin V motor complex, respectively.

Kinase inhibitors are playing an increasing role in the oncologist's pharmacopeia. They induce a death that can almost uniformly be inhibited by BCL-2 or related antiapoptotic proteins, designating the intrinsic apoptotic pathway as the key arbiter of cell death downstream of kinase inhibition. BIM protein levels increase in cancer cells that are sensitive to inhibitors of the epidermal growth factor receptor. This increase is due to increased transcription, but the details regarding this transcriptional control remain to be elucidated.

6. THE BCL-2 FAMILY AND CANCER

Even before anything was known about its role in controlling cell death, it was evident that BCL-2 played a role in cancer. BCL-2 first caught the attention of molecular biologists solely due to its location at the breakpoint of the t(14;18) translocation present in nearly all cases of follicular lymphoma, an indolent malignancy of germinal center B-lymphocytes. This translocation placed the BCL-2 gene on chromosome 18 under the control of the heavy chain promoter on chromosome 14, resulting in high levels of BCL-2 expression in cells in the B-lymphocyte lineage. Subsequent to its cloning, numerous experiments indicated its role in inhibiting cell death and its ability to act as an oncogene.

High levels of BCL-2 expression in cancer may be found most consistently in the lymphoid cancers follicular lymphoma and chronic lymphocytic leukemia (CLL). The presence of a t(14;18) is very rare in CLL, however. The loss of miR15 and miR16, micro-RNA loci that can suppress BCL-2 mRNA levels, may be responsible for increased BCL-2 expression in some cases of CLL, but it is not clear what proportion. Of the antiapoptotic proteins, levels of BCL-2, MCL-1, or BCL-XL have been best examined in cancer. One or more of these proteins may be found in a very wide range of cancers of all kinds. Much depends on the level of detection, and in most cases, it is not possible to know the level of expression once a certain threshold detectable by immunohistochemistry is reached. When expression of antiapoptotic protein is compared with clinical outcomes, the record is quite mixed across cancers, with certain studies showing inferior prognosis with higher antiapoptotic protein expression and others showing superior prognosis.

At first, it might be expected that expression of antiapoptotic proteins would universally confer clearly inferior prognosis. After all, antiapoptotic proteins like BCL-2, when overexpressed in cancer cell lines in vitro, induce resistance to a very wide variety of types of chemotherapy and radiation. However, it is very important to consider the basis of expression in cell culture models in vitro versus cancer cells in vivo. In most cell culture studies of BCL-2, a cell line that is growing well is supplemented by extra BCL-2 via forced over-expression. In this case, the BCL-2 is very likely to provide extra antiapoptotic reserve and promote resistance to apoptotic signaling from applied toxins. In the case of a cancer cell in vivo, however, BCL-2 can be selected for, but not over-expressed in the expectation of a subsequent chemotherapy treatment. The selection pressure for increased antiapoptotic protein expression can be driven by nearly ubiquitous cancer phenotypes such as genomic instability and oncogene activation. The subsequent death signaling, ultimately conducted by proapoptotic proteins, can be blocked by expression of antiapoptotic proteins like BCL-2, which can bind and sequester the proapoptotic proteins. But BCL-2 in this instance is now unable to sequester subsequent additional proapoptotic signaling. Indeed, the BCL-2 is now primed with pro-death proteins that can be released to kill the cell should BCL-2 function be abrogated in any way. To simplify, in the case of the over-expression cell culture model, the excess BCL-2 is largely “empty,” whereas in the case of the cancer cell, it is largely “full” (Figure 5-3).

In consequence, overexpression of BCL-2 that is concurrently laden with pro-death proteins may in fact predispose to chemosensitivity. For example, follicular

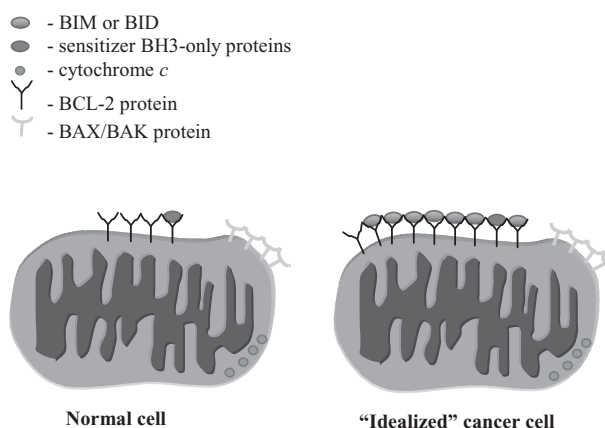


Figure 5-3. Illustration representing an unprimed mitochondrion versus a primed mitochondrion. Although it may express more BCL-2 than the normal mitochondrion, the primed mitochondrion has less antiapoptotic reserve as a result of significant priming by activator BH3-only proteins. With permission from Springer Science+Business Media: Del Gaizo Moore V and Letai A. Rational design of therapeutics targeting the BCL-2 family: are some cancer cells primed for death but waiting for a final push? *Adv Exp Med Biol.* 2008;615:159–75 (Figure 3), Copyright © 2008. See Color Plate 7.

lymphoma and CLL express very high levels of BCL-2 but are also extremely chemosensitive. Although they are difficult to cure permanently, initial treatment of either disease with modern chemotherapy is usually rewarded by a complete response with no evidence of residual disease. Therefore, the expression of antiapoptotic proteins alone is usually insufficient to predict the response of the mitochondrial apoptotic pathway to death signaling from chemotherapy. Instead, strategies that can simultaneously weigh the input of all anti- and proapoptotic BCL-2 family proteins are needed. One such strategy is BH3 profiling, which uses synthetic BH3 domain peptides to apply standardized death signals to mitochondria so that the readiness of a mitochondrion to undergo apoptosis can be objectively measured in a controlled fashion.

Of course, once subjected to chemotherapy, cancer cells may select for higher BCL-2 expression, and increased BCL-2 may indeed be an important source of secondary chemoresistance in clinical cancer therapy. However, the longitudinal studies comparing protein expression in de novo chemosensitive tumors with that in relapsed and chemorefractory samples from the same patients have not been performed. Therefore, the highly plausible hypothesis that overexpression of BCL-2 family proteins can contribute to acquired chemoresistance in cancer in vivo must still be considered formally unproven.

However, the abundant evidence from preclinical studies of the potential for BCL-2 and related antiapoptotic proteins to confer chemoresistance has fostered

considerable enthusiasm for the clinical targeting of BCL-2. At this writing, at least four individual molecules that target BCL-2 have entered clinical trials. As these trials mature and progress into combinations with conventional chemotherapy agents, the importance of BCL-2 in promoting chemoresistance and supporting cancer cell survival will be tested.

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6

Endoplasmic Reticulum Stress Response in Cell Death and Cell Survival

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1. INTRODUCTION

The endoplasmic reticulum (ER) serves as the primary cellular protein processing factory where polypeptides destined for secretion or membrane insertion are folded. This membrane-bound organelle recruits translating ribosomes, translocates newly synthesized peptides into its lumen, and promotes a variety of post-translational modifications and chaperone-facilitated folding events. Additionally, in higher eukaryotes ER serves as the major intracellular Ca^{2+} store. Because the ER encompasses about half the total membrane area and one-third the newly translated proteins in a typical eukaryotic cell, its proper function is critical for numerous aspects of cell physiology, including vesicle trafficking, lipid and membrane biogenesis, and protein targeting and secretion. Accordingly, metazoan cells react rapidly to ER dysfunction through a set of adaptive pathways known collectively as the ER stress response (ESR).

The ESR can be triggered by disparate perturbations in normal ER function, such as the accumulation of unfolded, misfolded, or excessive protein; ER lipid or glycolipid imbalances; or changes in the redox or ionic conditions of the ER lumen. In response to such dysfunction, the ESR acts both to increase the capacity of the ER to fold and process client proteins and to alleviate the burden on the organelle by reducing the amount of protein inside the ER.

These effects are achieved through four major pathways: (1) The unfolded protein response (UPR), a transcription-dependent induction of ER luminal chaperone proteins (and many other components of the secretory apparatus) that augments the polypeptide folding and processing capacity of the ER; (2) the control of protein translation to modulate the polypeptide traffic into the ER; (3) the activation of proteasome-dependent

ER-associated degradation (ERAD); and (4) induction of autophagic protein degradation to remove excess ER proteins. Normally, this suite of responses succeeds in restoring ER homeostasis. However, in metazoans, persistent or intense ER stress can also trigger programmed cell death, or apoptosis.

ER stress and the apoptotic program coupled to it have been implicated in many major human pathologies, including diabetes, obesity, neurodegenerative disorders, viral infection, and a variety of ER storage diseases. However, the initiation and execution steps of ER stress-induced apoptosis in mammals remain poorly understood. Classical genetics has facilitated the detailed dissection of the ESR in lower organisms such as *Saccharomyces cerevisiae*, but the greater complexity and intractable genetics of the mammalian pathways mean that there remains much to discover.

2. THE ESR IN YEAST

The foundation of our knowledge of the ESR rests on the genetic analysis of the budding yeast *S. cerevisiae*. In yeast, ER stress is monitored by Ire1p, an ER transmembrane protein with an N-terminal luminal domain and cytoplasmic C-terminal serine/threonine kinase and endonuclease domains. Normally, the ER luminal domain of a single Ire1p molecule interacts with Kar2p, an ER luminal chaperone of the Hsp70 family that assists in folding nascent polypeptides entering the lumen. Under ER stress conditions unfolded or misfolded proteins accumulate inside the ER and titrate Kar2p away from Ire1p. The removal of Kar2p permits the oligomerization of Ire1p through its luminal domain and the subsequent activation of Ire1p by *trans*-autophosphorylation via the C-terminal kinase domain.

Recent structural studies of the Ire1p luminal domain indicate that the induction of Ire1p activity by unfolded protein may be more complex than previously appreciated. Indeed, the crystal structure of the core luminal domain of yeast Ire1p revealed that the interface between Ire1p dimers forms a groove reminiscent of mammalian major histocompatibility complex (MHC) proteins, suggesting that Ire1p may bind directly to unfolded proteins. Furthermore, structure-guided mutations in amino acids at the dimer interface suggest that this interaction is required for Ire1p to assemble into higher-order oligomers that are necessary for UPR activation. On the other hand, the crystal structure of the luminal domain of human Ire1p homolog IRE1 α (see [Section 3](#)) shows that, although the large dimer interface and MHC-like groove are present, the groove in the mammalian protein is too narrow to accommodate peptide binding. Because of this potential discrepancy, it will be interesting to learn whether genetic experiments confirm a physiologic role for direct binding of unfolded proteins by Ire1 proteins in yeast and mammals.

Once activated, Ire1p signals to the nucleus to initiate the transcription-dependent UPR. The endonuclease domain of activated Ire1p splices the mRNA of the HAC1 gene, which encodes a bZIP transcription factor that drives expression of UPR-responsive genes. HAC1 mRNA is constitutively transcribed under resting conditions but contains an intron with secondary structure that blocks translation. During ER stress, activated Ire1p splices out the translation attenuator intron from the HAC1 message in a novel reaction independent of the spliceosome. The HAC1 mRNA fragments are then ligated together by the tRNA ligase, Trl1p. Splicing removes 252 nucleotides near the 3' end of the HAC1 transcript and replaces the last 10 codons of the open reading frame with a new 18-amino acid exon but does not affect the N-terminal DNA binding domain of Hac1p. This C-terminal 18-amino acid segment is a potent transactivation domain, significantly enhancing Hac1p-mediated upregulation of UPR target genes. Therefore, Ire1p-mediated splicing both facilitates HAC1 mRNA translation and adds a more powerful transactivation domain, allowing efficient UPR induction by mature Hac1p.

Mature Hac1p upregulates transcription of genes involved in all stages of the secretory pathway, including chaperones to assist protein folding in the ER lumen, enzymes involved in phospholipid synthesis and ER membrane biogenesis, and components of the protein export pathway. These targets of Hac1p reduce the burden of unfolded protein in the ER by increasing the capacity and efficiency of protein folding and export in the lumen. Remarkably, an estimated 5% of all yeast

genes are induced in response to ER stress, suggesting that the accumulation of unfolded proteins in the ER may remodel global cell physiology.

For years, it was thought that yeast lacked the second major ESR pathway, that of translational control. Recently, however, it has been revealed that Gcn2p, a kinase normally activated by uncharged transfer RNAs, phosphorylates the eukaryotic translation initiation factor 2 subunit α (eIF2 α) in response to ER stress. Phosphorylated eIF2 α leads to upregulation, via a translational mechanism, of the transcription factor Gcn4p. On its induction, Gcn4p stimulates the expression of amino acid biosynthesis and transport genes. Additionally, it is required for the full activation of a variety of UPR genes, demonstrating that a form of translational control exists in the yeast ESR.

ER stress in yeast also activates a proteolytic pathway known as ERAD. In ERAD, misfolded proteins in the ER membrane or lumen are actively retro-translocated, or "dislocated," into the cytoplasm, where they are substrates for ubiquitin-mediated degradation by the proteasome. ERAD can be divided into four conceptual steps: (1) the recognition and targeting of an unfolded substrate to the dislocation apparatus, (2) its transport across the ER membrane, (3) its release into the cytosol, and (4) its degradation. The components that mediate each step are not fully understood and appear to vary by substrate, including differences between soluble luminal and membrane proteins.

The recognition and targeting of irreparably misfolded polypeptides to the dislocation machinery are mediated, at least in part, by protein glycosylation and chaperone proteins in the ER lumen. In particular, chaperones such as Kar2p and protein disulfide isomerase 1 (PDI1) play an important role, at least in the case of soluble ERAD substrates. In some cases, glycosylation of misfolded proteins is also necessary for recognition by the ERAD machinery, possibly via a distinct set of chaperones. However, the precise reason for this requirement is not yet clear.

Some lines of evidence indicate that the dislocation step itself occurs, at least for some ERAD substrates, through the Sec61 channel, which also mediates the entry of nascent polypeptides into the lumen from ER-associated ribosomes. Both genetic and biochemical evidence suggest that several ERAD substrates require Sec61 function, implying that the channel can act bidirectionally. On the other hand, it is not clear whether all ERAD substrates require Sec61 to exit the ER, nor is there any mechanistic model for how soluble misfolded proteins in the lumen could be threaded into the Sec61 channel. Therefore, other means of ER egress may exist.

The release and ubiquitination of ERAD substrates are likely coordinated. Cdc48p, a cytosolic member of the ATPases associated with diverse cellular activities (AAA family of ATPases), participates in pulling ERAD substrates out of the ER in an adenosine triphosphate (ATP)-dependent manner, in cooperation with Ufd1p and Npl4p. In concert with Cdc48p action, ERAD substrates are ubiquitinated and targeted for degradation via the proteasome. Ubiquitin-conjugating enzymes participating in ERAD include Ubc1p, Ubc6p, Ubc7p, and its membrane-anchored binding partner Cue1p. Other proteins physically and genetically interact with the ubiquitin-conjugating machinery during dislocation, including Der1p, Der3p/Hrd1p, and Hrd3p, although the exact biochemical functions of all these components are not known. In addition to signaling for degradation, ubiquitination may assist in removing some substrates from the ER via a ratchet mechanism, whereby polyubiquitinated chains are prevented from passively sliding backwards through the Sec61 or other channel. Consistent with this model, inhibiting polyubiquitination causes the accumulation of ERAD substrates inside the ER.

Interestingly, ERAD and the UPR are reciprocally regulated. On the one hand, critical ERAD genes, such as DER1 and HRD3, are strongly induced by ER stress in wild-type but not *ire1Δ* yeast, indicating that the UPR can upregulate ERAD. On the other hand, in ERAD-defective strains, such as *ubc1Δ*, *ubc7Δ*, *der1Δ*, *hrd1Δ*, or *hrd3Δ*, the UPR is constitutively activated, indicating a feedback control of ERAD over the UPR. Therefore, in ERAD-deficient mutants, misfolded protein in the ER cannot be removed, causing the cells to activate the UPR to restore ER homeostasis. Importantly, single mutations impairing either the UPR or ERAD do not grossly affect yeast cell viability, but combining them results in synthetic toxicity. Thus ERAD and the UPR cooperate and the ESR is critical for cell viability, even in the absence of unusual stress.

Protein misfolding during ER stress can lead to the formation of protein aggregates that cannot be efficiently degraded by ERAD. Recently, autophagy, a catabolic pathway essential for cell survival during periods of starvation, has been shown to be induced and to play a protective function during the ESR. During autophagy, cytoplasmic constituents, including protein aggregates and organelles, are engulfed by double-membrane vesicles and degraded in a lysosome-dependent manner. During the ESR, autophagy is able to alleviate pathological expansion of the ER and increase cell viability, likely by degrading fragments of the ER, including the accumulated misfolded proteins and their aggregates. Consis-

tent with its important protective function during ESR, several genes necessary for the mediation and execution of autophagy, including ATG1, ATG8, ATG9, ATG16, VPS4, and PEP4, are critical for yeast cell survival during ER stress.

Activity of the essential autophagy kinase, Atg1p, is upregulated and necessary for the mediation of ESR-induced autophagy. The mechanism of this induction remains unclear but may depend of the function of Ire1p and Hac1p, as yeast strains deficient for either are unable to upregulate autophagy during ESR. Additionally, mRNA levels of many genes necessary for the mediation of autophagy, including ATG8, ATG14, and APE1, are transcriptionally upregulated after ER stress.

3. THE ESR IN MAMMALS

In mammals, many of the important features of the yeast ESR are conserved, but with additional complexities (Figure 6-1). Mammals have two homologs of Ire1p (IRE1 α and IRE1 β) and a homolog of Kar2p (BiP/Grp78), which serve as sentinels of ER stress and are believed to function similarly to the corresponding yeast proteins. IRE1 α is ubiquitously expressed, whereas IRE1 β is detected only in the intestine. Like yeast IRE1p, overexpression of mammalian IRE1 α activates the UPR in the absence of any ER stress signal. However, although IRE1p is absolutely required in yeast for initiation of the UPR, cells derived from Ire1 $\alpha^{-/-}$ /Ire1 $\beta^{-/-}$ embryos show no obvious UPR defect, suggesting existence of compensatory pathways, at least in this cell type.

The mammalian X-box-binding protein-1 (XBP-1), a bZIP member of the CREB/ATF family of transcription factors, serves as a functional homolog of yeast Hac1p. XBP-1 mRNA is ubiquitous in adult tissues but is preferentially expressed in fetal exocrine glands, osteoblasts, chondroblasts, and liver. Like HAC1, newly synthesized XBP-1 transcript must be spliced by activated IRE1 to give a mature mRNA. However, unlike HAC1, both the precursor and the spliced form of XBP-1 mRNA are translated. When activated by ER stress, IRE1 excises a 26-nucleotide intron from the immature XBP-1 transcript, resulting in a frame shift and the replacement of the C-terminal domain of the protein, analogous to the splicing of HAC1 mRNA. This results in a dramatic change in the properties of the encoded protein: although immature XBP-1 is unstable and represses UPR target genes, mature XBP-1 protein is stable and can efficiently drive UPR target gene transcription.

Unlike HAC1 mRNA, mammalian XBP-1 mRNA is not expressed at high levels in unstressed cells. Instead, the expression of XBP-1 mRNA is regulated by the

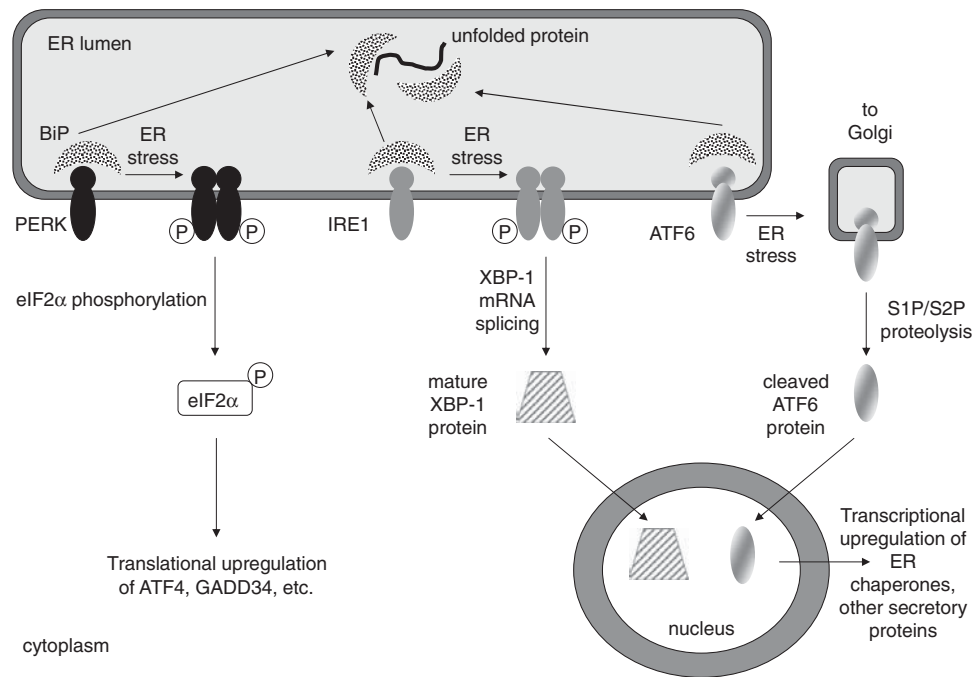


Figure 6-1. Mammalian ESR signaling. Unfolded protein in the ER lumen titrates BiP away from three sentinels of ER stress: PERK, IRE1, and ATF6. Activated PERK phosphorylates the translation initiation factor eIF2 α to slow global protein synthesis temporarily and upregulate certain stress-inducible messages, such as ATF4. Activated IRE1 splices the mRNA for XBP-1 to allow the translation of mature XBP-1 protein, a transcription factor that mediates the transcriptional upregulation of numerous genes involved in mammalian ER function and the secretory pathway in general. Similarly, during ER stress ATF6 traffics to the Golgi, where it is cleaved by S1P/S2P proteases and thereby released from the membrane to activate a distinct but overlapping set of genes in the nucleus. See text for details.

transcription factor ATF6, which is itself an important component of the ESR. Like HAC1 and XBP-1, ATF6 is activated by ER stress via an unusual mechanism. ATF6 is a type II transmembrane protein in the ER of unstressed cells. As with IRE1, the ER luminal domain of ATF6 interacts with BiP, which binds to a Golgi localization signal (GLS) sequence on ATF6. During ER stress, BiP is titrated away from ATF6, exposing the GLS and allowing ATF6 to traffic to the Golgi. In the Golgi, ATF6 is cleaved by the site-1 and site-2 proteases, the same enzymes that cleave membrane-bound sterol response element-binding proteins during conditions of sterol starvation. When ATF6 is cleaved, its N-terminal bZIP domain is liberated from the membrane and enters the nucleus to activate transcription of XBP-1 and other UPR target genes. The overall structure of ATF6 does not resemble Hac1p, and ATF6 protein levels are not regulated by mRNA processing. However, ATF6 does share significant sequence identity with Hac1p in its N-terminal basic region, and ATF6 over-expression activates many targets of the mammalian UPR. In some systems, ATF6 action precedes that of XBP-1 during ER stress, suggesting a time-dependent shift in the UPR transcriptional response. Other studies comprising reporters for

IRE1, ATF6, and protein kinase R-like endoplasmic reticulum kinase (PERK; see below) also show temporal control of ESR signaling, with the IRE1 branch attenuating significantly earlier than the ATF6 or, especially, PERK branch in response to pharmacological ER stress. ATF6 and IRE1/XBP-1 therefore play distinct but complementary and time-adjusted roles in adjusting UPR-mediated transcription during persistent stress.

The second major pathway of the mammalian ESR depends on the control of protein translation. As in yeast, translational control in the mammalian ESR is mediated by the phosphorylation of the translation initiation factor eIF2 α . EIF2 α is a tripartite guanosine triphosphate (GTP) hydrolyzing protein complex that plays a role in recruiting the initiator methionyl-tRNA to the 40S ribosome to begin mRNA translation. To initiate subsequent rounds of translation, eIF2 must exchange its bound guanosine diphosphate (GDP) for GTP, a process facilitated by the guanine nucleotide exchange factor (GEF), eIF2B. However, when the α subunit of eIF2 is phosphorylated on Ser51, it binds to eIF2B with much greater affinity. This interaction physically sequesters eIF2B, which is stoichiometrically limiting in the cell, and prevents it from mediating the exchange of GDP for

GTP by eIF2. Therefore, phosphorylated eIF2 α downregulates global translation by inhibiting its own GEF.

Mammals have four eIF2 α kinases that respond to diverse stress stimuli: (1) general control nonrepressed 2 (GCN2); (2) protein kinase R (PKR); (3) heme-regulated inhibitor (HRI); and (4) PERK. Like its counterpart in yeast, mammalian GCN2 is activated during amino acid starvation by binding to uncharged tRNAs. PKR is activated by double-stranded RNAs and is an important component of the interferon-mediated antiviral response. HRI is restricted to erythroid cells and is inhibited by heme, such that it blocks the translation of new globin chains when insufficient heme exists to assemble holo-hemoglobin. Finally, PERK phosphorylates eIF2 α in response to ER stress.

Like IRE1, PERK is a type I ER transmembrane protein. The large ER luminal domain of PERK is homologous to that of IRE1 α and IRE1 β , implying that its activation mechanism may resemble that of mammalian and yeast IRE1 proteins. Indeed, the luminal domains of both, IRE1 and PERK, interact with BiP under resting conditions but dimerize in a ligand-independent manner during ER stress. As in yeast, it is believed that the excess unfolded protein that accumulates during ER stress competes with mammalian IRE1 and PERK for BiP binding, allowing IRE1 or PERK to homodimerize and self-activate by *trans*-autophosphorylation. Consistent with this model, the displacement of BiP from IRE1 or PERK is correlated with the appearance of activated PERK and IRE1, and over-expression of BiP attenuates their activation.

The phosphorylation of mammalian eIF2 α (e.g., by PERK) downregulates the translation of most mRNAs. It has been suggested that this translation attenuation alleviates the burden on the stressed ER by transiently reducing the traffic of newly synthesized polypeptides into the organelle. Indeed, PERK $^{-/-}$ cells are hypersensitive to ER stress. Paradoxically, however, some components of the ESR are induced at the translational level when eIF2 α is phosphorylated, including the transcription factor ATF4 and its downstream target C/EBP homologous protein (CHOP).

Although ATF4 mRNA is constitutively expressed, it contains an inhibitory upstream open reading frame (uORF) that prevents efficient translation of the downstream gene. Accordingly, under resting conditions, ATF4 mRNA is associated with monoribosomes and low-molecular-weight polyribosomes, indicating that it is inefficiently translated. However, ATF4 mRNA quickly shifts to heavier ribosomal fractions and is selectively translated in a PERK-dependent fashion on ER stress induction. This is due to the fact that conditions

limiting eIF2 activity also increase ribosomal bypass scanning of the upstream uORF in the 5' leader to allow the preferential translation of ATF4 mRNA, even when most protein translation is halted. This mechanism is reminiscent of the upregulation of GCN4 mRNA in yeast by Gcn2p-mediated eIF2 α phosphorylation. Therefore, although eIF2 α phosphorylation inhibits the efficient translation of most mRNAs, it activates the translation of a small subpopulation of transcripts that contain small uORFs, such as ATF4.

ATF4 is not the only important gene subject to translational control in the mammalian ESR. Indeed, over-expression of ATF4 is not sufficient to activate the full complement of UPR genes, and the gross phenotype of ATF4 $^{-/-}$ mice is different from that of PERK $^{-/-}$ mice and eIF2 $\alpha^{A/A}$ knock-in mice, which harbor a homozygous Ser⁵¹→Ala⁵¹ mutation at the critical phosphorylation site in the endogenous eIF2 α locus. EIF2 $\alpha^{A/A}$ cells fail to upregulate about one-third of the genes normally induced by ER stress and are hypersensitive to ER stress-induced apoptosis, indicating that translational control affects a wide range of ER stress-responsive genes. However, eIF2 $\alpha^{A/A}$ cells also show transcription profile differences from wild-type cells under unstressed conditions, so the immediate consequences of the loss of eIF2 α phosphorylation during ER stress remain to be dissected in detail. On the other hand, ATF4 is critical for the full response to stresses that induce eIF2 α phosphorylation, probably through the direct transcriptional upregulation of UPR targets such as BiP and CHOP.

CHOP is a bZIP transcription factor that contains an ER stress response element in its promoter and is transcriptionally upregulated by ATF6. Interestingly, CHOP protein induction also stringently depends on eIF2 α phosphorylation, probably both because CHOP transcription is also activated by ATF4 and because the 5' leader of the CHOP mRNA contains small uORFs. CHOP can form heterodimers with members of the C/EBP and fos-jun families of transcription factors and likely contributes to the regulation of many genes in orchestrating the transcriptional component of the ESR.

EIF2 α signaling is also controlled by protein dephosphorylation mediated by the general cellular serine/threonine phosphatase protein phosphatase 1 (PP1). The catalytic subunit of PP1 shows little, if any, intrinsic substrate specificity and relies instead on the binding of nonenzymatic cofactors to direct its subcellular localization and substrate choice. The specific, stress-induced cofactor of PP1 directing it to dephosphorylate eIF2 α is GADD34. Like CHOP and ATF4, the expression of GADD34 itself is activated by eIF2 α phosphorylation,

and GADD34 induction requires GCN2 or PERK under conditions of amino acid starvation or ER stress, respectively. At least in some contexts, this may be mediated by upregulation of GADD34 mRNA by CHOP and/or the transcription factor ATF3, which is also induced by ATF4. Therefore, the regulation of eIF2 α by GADD34/PP1 completes a feedback loop to control translation: during ER stress, PERK phosphorylates eIF2 α , which induces the expression of ATF4, ATF3, and CHOP. These transcription factors then upregulate GADD34, which mediates the dephosphorylation of eIF2 α by PP1. In this way, protein translation returns to its baseline state after ER stress has abated. Consistent with this model, GADD34^{-/-} cells are impaired in their ability to resume normal translation after ER stress.

Other proteins can mediate eIF2 α dephosphorylation as well. A constitutively expressed, housekeeping homolog of GADD34, termed CREP, binds to PP1 and keeps eIF2 α dephosphorylated in the absence of stress. Indeed, RNAi against CREP induces eIF2 α phosphorylation under basal conditions. More recently, the SH2/SH3 domain-containing protein Nck-1 has been shown to antagonize PERK signaling in a phosphatase-dependent manner, probably through the direct dephosphorylation of activated PERK itself. Nck-1 activity also results in the dephosphorylation of eIF2 α , although whether it does so indirectly, by inactivating PERK, or directly, by mediating eIF2 α dephosphorylation (or both), remains to be determined.

The bZIP transcription factor Nrf2 was recently identified as a second substrate of PERK. On ESR induction, PERK phosphorylation causes Nrf2 to relocate from the cytoplasm to the nucleus, where it upregulates a range of antioxidant response genes. Therefore, a loss of Nrf2 function may partly explain why PERK^{-/-} cells experience increased oxidative stress in response to perturbations in ER function. Consistent with this model, Nrf2^{-/-} cells are sensitized to ER stress, providing another way in which PERK signaling promotes cell survival.

The mammalian ERAD pathway is less well understood than that of yeast, but several parallels are clear. As in yeast, glycosylation probably participates in the recognition of mammalian ERAD substrates, as do BiP and PDI. Similarly, at least some mammalian ERAD substrates may be dislocated through the Sec61 channel. Ubiquitination machinery similar to that of yeast is also employed, and a homolog of Cdc48p, termed p97, has been shown to cooperate with mammalian homologs of Ufd1p and Npl4p in substrate dislocation. Additionally, Derlin1, a mammalian homolog of Der1p, mediates the association of p97 with the ER membrane to facilitate ERAD.

As might be expected, mammalian ERAD pathways are more complex than those of yeast, and several novel components have been described. For example, the U-box carboxyl terminus of Hsc70-interacting protein (CHIP) has been recently identified as a ubiquitin E3 ligase that binds to chaperone and E2 enzymes to mediate ERAD. Interestingly, Parkin, a gene associated with a recessive form of juvenile Parkinson's disease (PD), encodes another ERAD ubiquitin E3 ligase. Parkin mediates the ERAD degradation of a novel form of α -synuclein, a protein also implicated in inherited forms of PD and the major constituent of Lewy inclusion bodies observed in the neurons of PD patients. Parkin disease mutants are unable to act on α -synuclein, suggesting that Parkin-mediated ERAD processing of α -synuclein is critical for avoiding PD. It seems likely that other substrate- and cell type-specific components of the mammalian ERAD pathway remain to be identified.

Recently, autophagy has been identified as an alternative protein degradation pathway that participates in the mammalian ESR. Autophagy is induced by a variety of ER stress compounds, including tunicamycin, thapsigargin, and brefeldin A, as well as by the accumulation of cytoplasmic protein aggregates in cells expressing polyglutamine repeats (polyQ). In most cases, inhibition of autophagy, through either use of pharmacological inhibitors or genetic ablation of critical genes, such as ATG5, ATG7, or beclin-1, leads to increased cell death after ER stress. This suggests a protective function of this catabolic pathway during the mammalian ESR. Additionally, autophagy participates in the removal of misfolded α 1-antitrypsin Z aggregates, which, when left unchecked, accumulate in the ER, leading to cytotoxicity. Similarly, defects in autophagy lead to the accumulation and formation of protein aggregates in the ER after expression of mutant form of the type-II transmembrane protein, dysferlin. This suggests a direct involvement of autophagy in degradation of at least some misfolded ER proteins and their aggregates. However, it is not yet clear to what extent autophagy contributes to the clearance of the accumulated ER proteins during the ESR, and the pro-autophagic signaling pathways downstream of ER stress remain highly controversial.

Several of the canonical ESR pathways have been implicated in the induction of autophagy after ER stress. As in yeast, the IRE1 pathway may be involved in autophagy during the ESR in mammalian cells. IRE1 $\alpha\beta$ -deficient mouse embryonic fibroblasts (MEFs) fail to upregulate autophagy after induction of ER stress but remain competent in starvation-induced autophagy. Interestingly, the kinase but not the RNase activity of IRE1 α is required for this function. In addition,

inhibition of JNK or dominant-negative TRAF2 prevents autophagy induced by ER stress, suggesting that IRE1 α controls autophagy through the recruitment and activating phosphorylation of TRAF2/JNK.

EIF2 α phosphorylation by GCN2 and PKR is known to be involved in the mediation of autophagy during starvation and herpes simplex virus (HSV) infection, respectively. Similarly, phosphorylation of eIF2 α by PERK has been suggested to mediate autophagy in response to ER stress induced by cytosolic accumulation of polyQ aggregates. In this system, autophagy is blocked by either expression of a dominant-negative form of PERK or the eIF2 $\alpha^{A/A}$ mutant protein, which cannot be phosphorylated. The PERK/eIF2 α pathway is also required for the upregulation of the autophagy mediator ATG12 at both the mRNA and protein levels in response to ER stress.

Finally, calcium flux perturbations occurring during ER stress have been suggested to participate in autophagy signaling. Consistent with the proposed role of calcium, autophagy induced in MCF-7 cells and hepatocytes by thapsigargin, an inhibitor of the ER Ca²⁺-dependent ATPase, is blocked by the calcium chelator BAPTA-AM. Influx of calcium into the cytosol in response to ER stress leads to the activation of the AMP-activated protein kinase (AMPK) through the Ca²⁺/calmodulin-dependent protein kinase kinase- β (CaMKK- β). AMPK, in turn, negatively regulates the mammalian target of rapamycin (mTOR) protein, leading to the induction of autophagy. Recently, the kinase PKC θ has also been shown to mediate thapsigargin-induced autophagy in immortalized hepatocytes. Interestingly, this appears to be independent of mTOR activity, suggesting that multiple independent pathways may mediate induction of autophagy during the ESR.

4. THE ESR AND CELL DEATH

The ESR acts to restore normal ER homeostasis and therefore is cytoprotective. However, when a stress is so strong or persistent that ER dysfunction cannot be corrected, metazoan cells can initiate apoptosis, allowing the regulated destruction of cells that are irreparably damaged or a risk to the organism as a whole. A unified model for ER stress-induced apoptosis is only beginning to emerge, but recent interest in the field has generated an increasing amount of information (see [Figure 6-2](#) for an overview).

Some core components of the ESR can function in ER stress-induced apoptosis as well. For example, mammalian Ire1 can activate JNK and other proapoptotic kinases such as ASK1, which may contribute to ER stress-induced apoptosis. ER stress inducers such as

tunicamycin, thapsigargin, or reducing agents, as well as the over-expression of IRE1 α , induce the activation of JNK. IRE1 also interacts with TRAF2, an adaptor protein involved in the signaling pathways of proinflammatory cytokines such as tumor necrosis factor α and interleukin-1. This interaction can recruit ASK1 to form an IRE1/TRAF2/ASK1 ternary complex, which in turn can activate JNK. The functional importance of JNK activation in the ER stress pathway has not been fully explored, but ASK1^{-/-} cells are partially resistant to ER stress-induced apoptosis, suggesting that JNK may promote apoptosis in this context.

CHOP has also been shown to promote apoptosis, and this effect can be blocked by BiP over-expression, suggesting that CHOP-activated apoptotic pathways are downstream from the ER. CHOP can transcriptionally downregulate the antiapoptotic protein Bcl-2 and upregulate DR5, a member of the death receptor protein family, two effectors that function in non-ER forms of cell death as well. Interestingly, CHOP also leads to a depletion of cellular glutathione and an increase of reactive oxygen species (ROS) in the ER, due in part to its induction of ERO1 α , an ER oxidase. Interfering with ERO1 α function reduces the accumulation of ROS in the stressed ER, leading to cytoprotection. This implies that ERO1 α may be an important apoptotic effector downstream of CHOP. CHOP^{-/-} MEFs are partially resistant to ER stress-induced cell death, although CHOP^{-/-} mice are not resistant to lethal doses of tunicamycin, suggesting that other proapoptotic pathways are also at work.

The eIF2 α phosphatase cofactors GADD34 and CReP also mediate apoptotic signaling downstream from the ER. During ER stress, GADD34^{-/-} cells display persistent eIF2 α phosphorylation and fewer misfolded protein aggregates in the ER lumen, suggesting that GADD34 function is proapoptotic. Indeed, GADD34^{-/-} mice are resistant to the toxic effects of tunicamycin. Similarly, RNAi-mediated knockdown of CReP protects cells from a variety of stimuli, including ER stress. It seems likely that the cytoprotection provided by the loss of GADD34 or CReP is due primarily to increased eIF2 α phosphorylation because enforcing eIF2 α phosphorylation with a constitutively active PERK has a similar effect.

ER stress can also directly activate well-known general regulators of mammalian apoptosis, including the Bcl-2 and caspase families of proteins. It has long been known that a pool of endogenous Bcl-2 resides in the ER membrane, and although Bcl-2 family members are thought to function principally at the mitochondrial outer membrane, there is ample evidence that they influence homeostasis and apoptosis from the ER as well. For example, variants of the antiapoptotic family

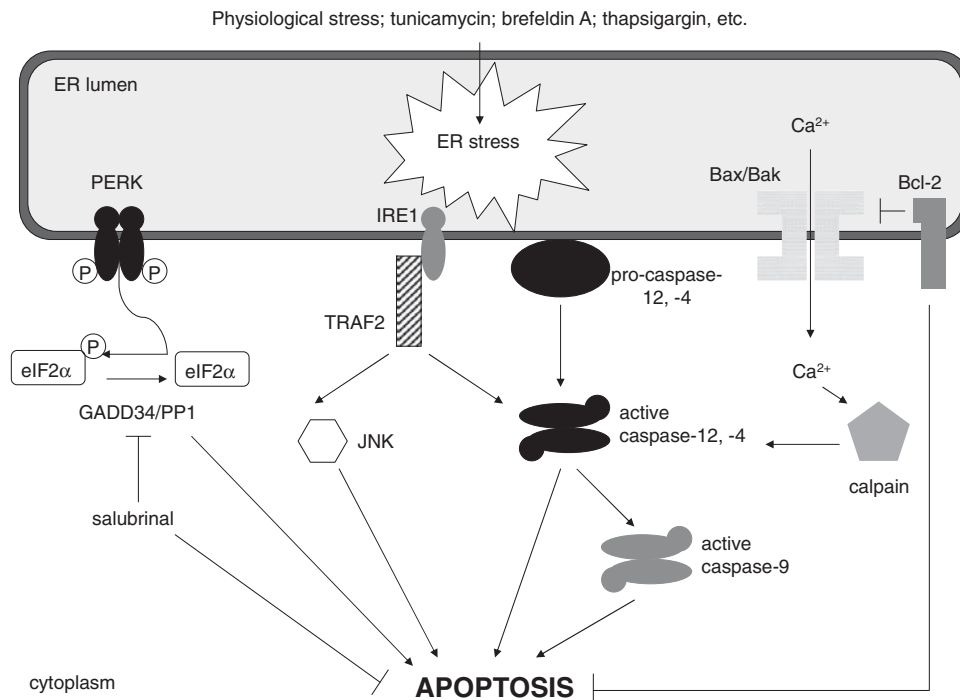


Figure 6-2. Simplified depiction of selected apoptotic pathways induced by ER stress. Physiologic or experimentally induced ER stress leads to the activation of PERK and, eventually, the GADD34/PP1 phosphatase complex, which dephosphorylates eIF2 α , promoting apoptosis. Genetic strategies or chemicals (e.g., salubrinal) that enforce eIF2 α phosphorylation protect cells from ER stress-induced apoptosis. Caspase-12 (mice) or -4 (humans) is associated with the cytoplasmic face of the ER membrane and can be activated by ER stress in several ways, including via IRE1 and TRAF2, or by cleavage by calpain, itself activated by the release of calcium from ER stores. Bcl-2 family members also reside in the ER membrane and influence apoptosis induced by ER stress, both through the regulation of calcium flux and amplification of the apoptotic signal via the mitochondrial pathway (not shown). See text for details.

members Bcl-2 or Bcl-X_L targeted specifically to the ER membrane can block apoptosis induced by pharmacological kinase inhibition or by proapoptotic Bcl-2 family members. Conversely, ER stress itself can upregulate or otherwise activate several BH3-only proapoptotic members of the Bcl-2 family, including Bim, BIK, and PUMA. Therefore, efferent signaling from the stressed ER can engage the Bcl-2 death machinery directly.

Recent studies have also demonstrated that the proapoptotic multidomain Bcl-2 family proteins, Bax and Bak, regulate ER stress-induced apoptosis. Bax^{-/-}/Bak^{-/-} MEFs are remarkably resistant to many forms of apoptosis, including ER stress, implying that ER stress and other apoptotic signaling pathways converge on Bax and Bak. Interestingly, endogenous Bax and Bak regulate ER stress-induced apoptosis from both the mitochondrial and ER membranes. In the ER membrane, Bax and Bak are crucial for maintaining the resting level of luminal calcium, probably through an interaction with the type 1 inositol triphosphate receptor. As a result, Bax^{-/-}/Bak^{-/-} cells display reduced calcium release from the ER in response to such stimuli as arachidonic acid and

oxidative stress, thereby attenuating apoptosis. However, in response to other ER stress stimuli, such as the ER-to-Golgi vesicle transport inhibitor brefeldin A, Bax and Bak must be present at both the ER and mitochondrial membranes for apoptotic execution to proceed normally. Therefore, Bax and Bak participate in signal integration between the ER and the mitochondria to influence cell survival choices from multiple locations within the cell. Indeed, more recently, Bax and Bak were found to interact directly with IRE1 α in the ER to promote UPR signaling, demonstrating a directly molecular connection between the ESR and the core apoptotic machinery.

The caspase family of proapoptotic cysteine proteases also plays a critical role in ER stress-induced apoptosis. Caspase-12, a murine protein associated with the cytosolic side of the ER membrane, is activated by ER stress-induced apoptosis, but not by non-ER stimuli, and is required for cell death in response to both pharmacological ER stress and ER-targeted Bim. Caspase-12 can be activated by ER stress in several ways. For example, the cytoplasmic calcium-activated protease calpain can cleave and activate caspase-12 in response to

calcium flux from the ER, which is often triggered by ER stress. Caspase-12 can also autoactivate through a direct association with IRE1 α and the adaptor protein TRAF2, though how the formation of this complex is regulated by ER stress is not yet clear. Caspase-12 has also been detected in high-molecular-weight complex with apoptosis-linked gene-2 protein and the ERAD mediator p97 (also referred to as valosin-containing protein). Interference with the formation of this complex protects cells from ER stress-induced apoptosis, presumably by blocking the activation of caspase-12. Because p97 is also involved in ERAD, it represents another protein that may mediate cross-talk between the prosurvival and proapoptotic pathways induced by ER stress.

Once activated, caspase-12 can initiate downstream apoptotic pathways. For example, ER stress can induce the activation of caspase-9 independent of Apaf-1, the usual mediator of caspase-9 activation. In addition, caspase-7 translocates to the ER in response to some apoptotic stimuli, and it has been proposed that caspase-7 can directly activate caspase-12. However, other experiments suggest that caspase-12 cleavage precedes caspase-7 cleavage under ER stress conditions, implying that the order of activation may be the opposite. In addition, glycogen synthase kinase (GSK) 3 β may influence caspase-3 activation specifically during ER stress, although whether this is a direct effect or a far-upstream event remains unclear.

It is worth noting that the role of human homologs of caspase-12 in ER stress-induced apoptosis has been controversial. However, it was recently shown that human caspase-4, which is 48% homologous to murine caspase-12, is localized to the ER membrane and is specifically activated by and required for ER stress-induced apoptosis. These data suggest that caspase-4 is the human functional counterpart of murine caspase-12.

Autophagy is generally a protective response against cellular stresses, but under some circumstances, especially when apoptosis is blocked, it can also contribute to cell death (referred to as type II programmed cell death). In fact, it has been a subject of much debate whether autophagy plays a pro-death or prosurvival function during ESR. It is clear that autophagy is cytoprotective during yeast ESR. Under most circumstances, this seems to be the case in mammalian cells as well. For example, ER stress-induced caspase-12 activation and cell death are enhanced in ATG5-deficient MEFs and in MEFs treated with the type III PI3 kinase inhibitor 3-methyladenine (3-MA), a potent inhibitor of autophagy. In contrast, autophagy may contribute to the cell death process in apoptosis-resistant cells, as ATG5-deficient

MEFs expressing Bcl-XL are more resistant to ER stress-induced cell death than wild-type MEFs expressing Bcl-XL. In addition, the influence of autophagy on the cell survival after ER stress may also depend on whether the cells have been transformed, as primary ATG5-deficient MEFs have been reported to be less sensitive to ER stress-induced cell death, and 3-MA protects nonimmortalized normal human colon cell line CCD-18C against ER stress toxicity.

5. THE ESR IN DEVELOPMENT AND TISSUE HOMEOSTASIS

In mammals, several components of the ESR are essential for organismal development and tissue homeostasis. As in yeast, this likely reflects the fact that normal physiologic fluctuations can strain the capacity of the ER such that cells need the ESR to cope with the secretory burden. As might be expected from this model, professional secretory cell types, such as plasma B cells and pancreatic β -islet cells, are among the most severely affected when the ESR is compromised.

Ire1 $\alpha^{-/-}$ mouse embryos die around day 10.5 of gestation as a result of unknown causes, but Ire1 $\alpha^{-/-}$ MEFs exhibit no obvious defect in the UPR. It may be that some embryonic cell types rely on IRE1 α for UPR signaling even though fibroblasts do not, or that IRE1 α is important to non-UPR functions in the embryo as well. On the other hand, mice deficient for IRE1 β restricted to the intestines develop without obvious abnormalities but display increased sensitivity to colitis.

Further downstream in the UPR, XBP-1 is essential for fetal hepatocyte growth. XBP-1 $^{-/-}$ embryos have hypoplastic livers with reduced cell proliferation and increased apoptosis and show reduced hematopoiesis that results in severe anemia and death. Whether this phenotype is due solely to the role of XBP-1 in the ESR is not yet clear. However, XBP-1 is also known to play an important role in plasma B cell differentiation, as XBP-1 $^{-/-}$ lymphocytes transplanted into RAG-2 chimeric mice display a severe defect in the generation of plasma cells. XBP-1 coordinates a broad transcriptional program in the developing B cell, upregulating many genes in the secretory pathway and the physical expansion of the ER. These data suggest that the XBP-1-mediated component of the UPR is critical for the ability of certain secretory cell types to develop and function.

The translational control pathway of the ESR also plays a prominent role in development. PERK is highly expressed in the pancreatic acini that secrete digestive enzyme, and in the islets of Langerhans, which produce and secrete the polypeptide hormones insulin and glucagon. Active, phosphorylated PERK can be

detected in the wild-type pancreas, lung, liver, spleen, and thymus. Furthermore, the normal phosphorylation of eIF2 α is lost in the PERK^{-/-} pancreas and thymus, suggesting that translational control downstream of the ER normally operates in those organs. Consistent with this hypothesis, compensatory activation of IRE1 α is detected in the pancreas of PERK^{-/-} mice.

PERK^{-/-} mice survive to birth but develop progressive disturbances in glucose metabolism and hypoinsulinemia. Although the neonatal pancreas of PERK^{-/-} mice is largely normal, the number of insulin-producing cells decreases over time and is associated with a profound increase in apoptosis in the pancreas. Therefore, PERK-mediated translational control is necessary to manage the processing and export of secreted proteins such as insulin under normal physiologic conditions, and inappropriate apoptosis and disease can occur when this aspect of the ESR is impaired.

Similar conclusions can be drawn from eIF2 α ^{A/A} mice, which exhibit a more severe phenotype. At birth, eIF2 α ^{A/A} mice are indistinguishable from their wild-type or heterozygous littermates, but most die within 18 hours as a result of hypoglycemia. Indeed, the induction of gluconeogenic enzyme genes in the liver is significantly reduced. Because fetal glycogen synthase expression is normally induced to promote the storage of maternal glucose as liver glycogen for survival during the early neonatal period, the reduced ability to store glycogen may contribute to the hypoglycemia in eIF2 α ^{A/A} neonates. In addition, insulin levels in the eIF2 α ^{A/A} pancreas are significantly lower than that of wild type.

The severe defects in glucose and insulin metabolism in PERK^{-/-} and eIF2 α ^{A/A} mice suggest that ER protein folding and the regulation of eIF2 α phosphorylation are important homeostatic controls in glucose and glycogen metabolism. Because eIF2 α ^{A/A} mice exhibit an earlier and more severe phenotype than PERK^{-/-} mice, additional eIF2 α kinases may participate in normal fetal and neonatal development. However, none of the single knockouts of the other three eIF2 α kinases exhibits any abnormality in glucose metabolism, suggesting that two or more of these enzymes in combination may be responsible for liver glycogen regulation.

Interestingly, mice with targeted deficiencies in genes involved in ER stress-induced apoptosis, including caspase-12, CHOP, EF2K, and BIK/Blk, generally display no obvious deleterious phenotype, confirming that the main homeostatic function of ESR lies in its ability to adjust cellular function to accommodate increased secretory burden, rather than in mediation of cell death in response to ER stress.

6. THE ESR IN HUMAN DISEASE

Dysregulation of the ESR or the apoptotic pathways coupled to it have been implicated in myriad human diseases. Three broad classes of diseases involving ER stress – diabetes, neuronal ischemic injury, and viral infection – are addressed briefly here as examples.

ER stress can contribute to both insulin-dependent (type 1) and insulin-resistant (type 2) diabetes. Interestingly, defects in the PERK-dependent eIF2 α phosphorylation pathway cause a disease in humans, known as Wolcott-Rallison syndrome (WRS), which is reminiscent of the phenotype of PERK^{-/-} mice. WRS is a rare, autosomal-recessive disorder characterized by permanent neonatal or early infancy nonautoimmune type 1 diabetes, with epiphyseal dysplasia, osteoporosis, and growth retardation occurring later. Mutations in the eukaryotic translation initiation factor 2- α kinase 3 gene (EIF2AK3), which encodes the human homolog of PERK, are the underlying genetic defect in WRS families. WRS-associated mutations in EIF2AK3 abrogate the eIF2 α kinase function of human PERK, confirming the involvement of the ESR in WRS. These findings have led to the suggestion that defects in other aspects of the ESR may contribute to type 1 diabetes, or that polymorphisms in the EIF2AK3 gene may predispose some non-WRS patients to develop type 1 diabetes.

More recently, ER stress has also been implicated in type 2 diabetes. Both genetic and diet-induced obesity in mice are associated with the activation of the ESR, possibly due to an increased burden on the cells of secretory organs. Interestingly, activation of the ESR antagonizes insulin signaling in both mouse models via the JNK-mediated phosphorylation of insulin receptor substrate 1. Furthermore, enforcing the expression of XBP-1 to increase UPR activation resulted in less ER stress and restored insulin sensitivity and proper glucose homeostasis. Intriguingly, “chemical chaperones” – or pharmacological agents that promote protein folding and ameliorate consequent ER stress – reduce hyperglycemia, tissue insulin sensitivity, and fatty liver disease in obese and diabetic mice. Therefore, ER stress may be a major upstream cause of diabetic symptoms in some contexts. Consistent with this notion, XBP-1^{+/-} mice are more susceptible to obesity-induced diabetes than were wild-type mice. Mutations or obesity-induced dysfunction in the UPR component of the ESR may therefore be a risk factor for type 2 diabetes in humans as well.

Interestingly, recent work suggests that ESR also contributes to lipid and carbohydrate metabolism in the liver. XBP-1 is induced in the livers of mice fed a high-carbohydrate diet, in conjunction with genes involved

in fatty acid biosynthesis. Conversely, deletion of XBP-1 in the liver results in a reduction of hepatic lipid biosynthesis and, secondarily, hypocholesterolemia and hypotriglyceridemia. Similarly, recent work also showed that eIF2 α dephosphorylation in the liver enhances glucose tolerance and steatosis in mice. Although not directly linked to diabetes per se, these newly discovered functions of core ESR components indicate that the ESR may integrate signals from various nutritional cues in the body to control the physiologic response to dietary carbohydrates and lipids. Therefore, dysfunctional ESR signaling may contribute to a range of metabolic disorders.

Activation of the ESR in acute ischemia/reperfusion (I/R) brain injury is also well documented. It has long been known that protein synthesis is inhibited in ischemic neurons on reperfusion and that translation gradually resumes in regions resistant to damage while remaining suppressed in vulnerable neurons. Phosphorylation of eIF2 α and inhibition of translational initiation are also found in I/R-injured brains. Some canonical downstream consequences of eIF2 α phosphorylation, such as the induction of ATF4, CHOP, and GADD34, are also observed, indicating that the translational control pathway of the ESR may be activated in toto. However, the protein synthesis block can persist even after eIF2 α has been dephosphorylated, suggesting that other inhibitory mechanisms exist.

EIF2 α phosphorylation in I/R injury is likely caused by a strong and rapid activation of PERK. Consistently, PKR $^{-/-}$, HRI $^{-/-}$, and GCN2 $^{-/-}$ mice show no reduction in I/R-induced eIF2 α phosphorylation, implying that PERK is solely responsible for this activity. However, the perinatal lethality of PERK $^{-/-}$ mice precludes testing them directly in an I/R model.

Exactly how I/R might activate PERK is not clear. Protein aggregates are observed inside I/R injured neurons, suggesting that perturbation of protein folding may play a role. Another possibility is that ROS accumulation in the ER leads to PERK activation. It has also been proposed that disturbances in ER calcium homeostasis, which are common in I/R injury, may cause ER stress in reperfused neurons. Indeed, inhibitors of ER calcium release, such as dantrolene or TMB-8, can protect cells from ischemic or excitotoxic injury. However, it is not yet clear whether eventual neuronal death is due to the loss of ER luminal calcium per se or the resultant spike of calcium in the cytoplasm (or both).

More recently, I/R injury has been found to induce other components of the ESR. Although activation of ATF6 is not observed, IRE1 is activated and XBP-1 is spliced and translated soon after reperfusion. Furthermore, caspase-12 activation occurs in both transient and

permanent models of focal ischemia, suggesting that ER-specific apoptotic pathways are also engaged. Caspase-12 activation might be an important, general proapoptotic event in I/R, as caspase-12 $^{-/-}$ mice are resistant to cell death in a related model of cardiac ischemia. However, it is not yet clear whether the activation of other ESR pathways is cytoprotective or cytotoxic in I/R injury. Selective pharmacological manipulation of certain ESR components may help to resolve this question.

As obligate intracellular parasites, viruses enforce the production of large amounts of polypeptides by cellular machinery and therefore place a heavy burden on organelles sensitive to protein overload, such as the ER. In particular, certain classes like the flaviviruses, which include important human pathogens such as the hepatitis C, dengue, yellow fever, and West Nile viruses, use the ER as the primary site for polyprotein processing, envelope glycoprotein biogenesis, and virion formation. Such ER-tropic viruses can perturb normal ER homeostasis and engage the ESR. Indeed, the protein products of the vesicular stomatitis virus (VSV), Sindbis, rabies, hepatitis C virus (HCV), the neurovirulent murine FrCasE virus, and the measles virus (MV) are all found in specific complexes with ER luminal chaperones. In some cases, interaction between the host chaperones and the viral proteins is essential for viral protein processing and virion assembly. In addition, a glut of viral glycoproteins in the ER may compete with cellular proteins for chaperones, leading to ER stress and the activation of the ESR.

In fact, ESR activation has been observed in many instances of viral infection. For example, Japanese encephalitis virus causes the hypertrophy of the ER membrane and induces such canonical UPR targets as BiP, calnexin, Grp94, and CHOP. Similarly, MV infection upregulates myriad ESR targets, including BiP, calreticulin, calnexin, Grp94, CHOP, and ATF4, implying the activation of both the UPR and PERK pathways. HCV also induces the cleavage and activation of ATF6, followed by upregulation of BiP and CHOP. Furthermore, the induction of BiP by HCV infection is dependent on the ESR elements in the BiP promoter, indicating an ESR-specific signal. Interestingly, the HCV E2 protein, which resides in the ER membrane, causes ER stress when expressed at low levels but, at higher levels, binds to and inhibits PERK, presumably to block eIF2 α phosphorylation and allow viral protein synthesis to continue.

ER stress-induced apoptosis is also observed during viral infection. A cytopathic strain of bovine viral diarrhoea virus (BVDV) activates PERK and eIF2 α , upregulates proapoptotic molecules such as CHOP and caspase-12, and downregulates Bcl-2. Therefore, ER stress may be the primary proapoptotic stimulus that

kills BVDV-infected cells. Similarly, respiratory syncytial virus (RSV) infection causes ER stress, the cleavage and activation of caspase-12, and apoptosis in a lung epithelial cell line. An antisense construct against caspase-12 reduced the number of apoptotic cells by almost four-fold, suggesting that caspase-12 is a critical component of RSV-induced apoptosis. Similarly, paramyxovirus induces the ESR, caspase-12 activation, and apoptosis, which can be blocked by a small molecule pan-inhibitor of all caspases, but not by specific inhibitors of non-caspase-12 family members, indicating that an ER stress-specific pathway is responsible for apoptosis in this system. An ER-specific apoptotic program may also be activated by hantavirus infection.

Although it is likely that ER stress is a common consequence of viral infection, it is not yet clear what role the resulting ESR may play in viral pathogenesis. Indeed, some components of the ESR, such as chaperone induction, might be expected to aid virus replication by facilitating the assembly of viral glycoproteins, while other pathways, such as PERK and caspase-12 activation, may hamper viral protein production or kill host cells, limiting the infection. Consistent with this idea, PERK is required for resistance to VSV infection, at least in cell culture. Additionally, HCV suppresses the IRE1/XBP-1 pathway, suggesting that blocking the UPR-driven induction of ERAD genes may promote viral replication by avoiding the ERAD-mediated degradation of viral proteins. On the other hand, some viruses may use ESR as an immune evasion strategy, as observed with cytomegalovirus, which co-opts ERAD to destroy major histocompatibility proteins and elude T-cell detection.

HSV provides another interesting example of the interplay between viral pathogenesis and the ESR. Recently, HSV was shown to induce protein synthesis-dependent activation of PERK, which is potentiated in cells lacking PKR, the main infection-activated eIF2 α kinase. This result suggests that multiple eIF2 α kinases may cooperate in the response to HSV infection. Interestingly, HSV encodes ICP34.5, a homolog of GADD34 that binds to cellular PP1 and mediates eIF2 α dephos-

phorylation to allow viral protein synthesis to continue. Deletion of the gene encoding ICP34.5 prevents HSV from replicating in certain cell types, suggesting that eIF2 α phosphorylation is critical in restricting infection. PKR is the kinase primarily responsible for the cell-type restriction of ICP34.5-deficient strains. However, if HSV infection also activates PERK, the ESR may partly compensate for PKR loss, and so ICP34.5 might be required to antagonize both PERK and PKR in some contexts. In either case, eIF2 α phosphorylation is likely the critical antiviral effect of PERK and PKR activation, because salubrinal, a small-molecule inhibitor of eIF2 α dephosphorylation, inhibits HSV replication in wild-type but not eIF2 $\alpha^{\Delta/\Delta}$ cells. In the future, it will be important to ask how the deletion of such ESR components as IRE1, XBP-1, PERK, CHOP, and caspase-12 affects the progression of viral infection in cell culture and animal models.

7. CONCLUSION

In a typical yeast cell, more than 1,500 newly synthesized polypeptides enter the ER per second. By extension, in mammalian cells specialized for protein secretion, such as plasma B cells or pancreatic β -islet cells, the flood into the ER is torrential. Because of this centrality of the ER to cell homeostasis, a thorough understanding of the ESR is important for any systems-level model of cell or organismal biology. Indeed, an emerging theme of the recent research in the field is that normal levels of protein flux into the ER can trigger the ESR, and so ESR components are critical for maintaining cell and organ homeostasis, even in the absence of any unusual stress. In addition, because metazoan cells can apoptose in response to ER stress, and because ER dysfunction and ER stress-induced apoptosis are implicated in many important human pathologies, an improved understanding of the ESR may facilitate the development of methods to manipulate these pathways for therapeutic benefit. Therefore, continuing research on ESR pathways promises to yield important basic and clinical insights far into the future.

7

Autophagy – The Liaison between the Lysosomal System and Cell Death

Hiroshi Koga and Ana Maria Cuervo

1. INTRODUCTION

The involvement of lysosomes, the organelle with the highest concentration of hydrolases, in cellular death has been extensively analyzed in different contexts in the past. In many of those studies, lysosomes were proposed to play a “passive” role in the cellular death process, resulting from the leakage of potent lysosomal enzymes into the cytosol. In fact, rupture of the lysosomal membrane after various types of cellular injury or under certain pathological conditions can lead to both apoptotic and nonapoptotic cell death. For example, lysomotropic agents, certain lipid products such as sphingosine or ceramide, a wide variety of death stimuli such as death receptor activation, p53 activation, microtubule-stabilizing agents, oxidative stress, and growth factor deprivation induce lysosomal permeabilization and the release of lysosomal proteases, generically known as cathepsins, into the cytosol. Studies using both genetic and pharmacological blockage of cathepsins support that cytosolic release of these lysosomal hydrolases can mediate caspase-dependent and -independent cell death.

The involvement of lysosomes in cellular death has recently been revisited, and a more active role for this organelle has been proposed. The main drive for this re-evaluation has been the recent advances in our understanding of one of the basic lysosomal functions: autophagy, or the self-digestion of intracellular components by lysosomes. In contrast to the “passive” role in cell death in which lysosomes are merely the carriers of the damaging enzymes that are released into the cytosol, in recent years a direct contribution of lysosomes as intact and properly functioning organelles to cell death has been proposed. The concept of autophagic cell death, however, is not new for cell death researchers.

In fact, this term has been extensively used in the classification of programmed cell death types based on morphological features (Figure 7-1). Thus, whereas in cell death type I or apoptotic cell death, the signature features are condensation of the nucleus and cytoplasm, DNA fragmentation, and early collapse of cytoskeletal elements, but preservation of organelles until late stages, the term cell death type II, or autophagic cell death, has been reserved for dying cells in which the presence of autophagosomes and autophagolysosomes – vesicular compartments related to autophagy – are the predominant morphological feature. In this case, early degradation of organelles but preservation of cytoskeletal elements until late stages are the signature features.

This connection between autophagy and cell death has come more as a shock to autophagy researchers, because most studies support a role for autophagy in sustaining cell survival. In this chapter we review some of the recent advances in the understanding of autophagy resulting from the better molecular characterization of this pathway and how the ability to genetically and pharmacologically modulate this lysosomal pathway has shed light on this apparent paradox.

2. AUTOPHAGY

Degradation of intracellular components is essential for maintenance of cellular homeostasis, continuous renewal of the proteome and organelles, removal of damaged cellular constituents, and the cellular response to environmental stressors. Two major proteolytic systems participate in intracellular protein clearance: (1) the ubiquitin/proteasome system, and (2) the lysosome. This degradation of intracellular proteins and organelles within lysosomes is called autophagy, a process conserved from yeast to mammalian cells.

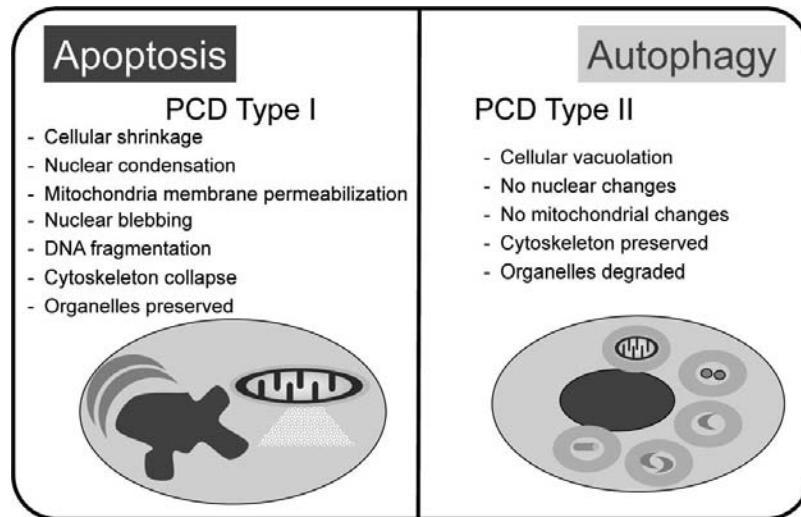


Figure 7-1. Morphological characteristics of different types of cell death. Cell death type I and type II is a common classification used based on the morphological characteristics of dying cells. Some of the distinctive characteristics are enumerated. PCD, programmed cell death.

2.1. Molecular dissection of autophagy

Different types of autophagy have been described on the basis of the mechanisms used for delivery of cargo to lysosomes, their regulation, and their intracellular functions. The three more common autophagic types in mammals are (1) macroautophagy, (2) microautophagy, and (3) chaperone-mediated autophagy (Figure 7-2).

Macroautophagy, quantitatively the most important type of autophagy, accounts for the degradation of both soluble proteins and organelles in lysosomes. Cargo is initially sequestered by an “isolation” membrane, originating from a pre-autophagosomal structure, or phagophore (Figure 7-2). This membrane elongates and fuses to form an autophagosome, a double membrane-bound vesicle that acquires the enzymes required for cargo degradation through fusion with secondary mature lysosomes in the cytosol. Approximately 30 novel gene products, known as Atg or autophagy-related proteins, participate in various macroautophagy steps. These proteins can be divided into four functional groups (Figure 7-3): (1) the initiation complex – a serine-threonine kinase complex formed by Atg1, Atg13, and Atg17 – that integrates signaling from the target of rapamycin (TOR)

kinase, which is a negative regulator of macroautophagy. This complex regulates the initiation, nucleation, and expansion steps of autophagosome formation); (2) the nucleation complex, a second kinase complex containing Beclin 1 (the mammalian ortholog of the yeast protein Atg6), hsVPS34 (a class III PI3K), and a particular subset of interacting proteins, required for the nucleation phase; (3) two ubiquitin-like conjugation systems, the Atg12/5 system and the LC3 (mammalian ortholog of the yeast protein Atg8), phosphatidylserine systems that act sequentially during autophagosome formation; and (4) a recycling system, controlled by Atg4 and Atg9 and responsible for the shuttling of Atg proteins onto and off of the autophagosomal

membranes during the formation of autophagosomes. The maturation of autophagosomes requires several GTPases (Rab 7 and Rab 24) and other factors involved in fusion events and vesicular trafficking. Macroautophagy has been classically considered an inducible type of autophagy, which becomes maximally active in response to starvation or stressors resulting in extensive intracellular damage. However, recent studies support the

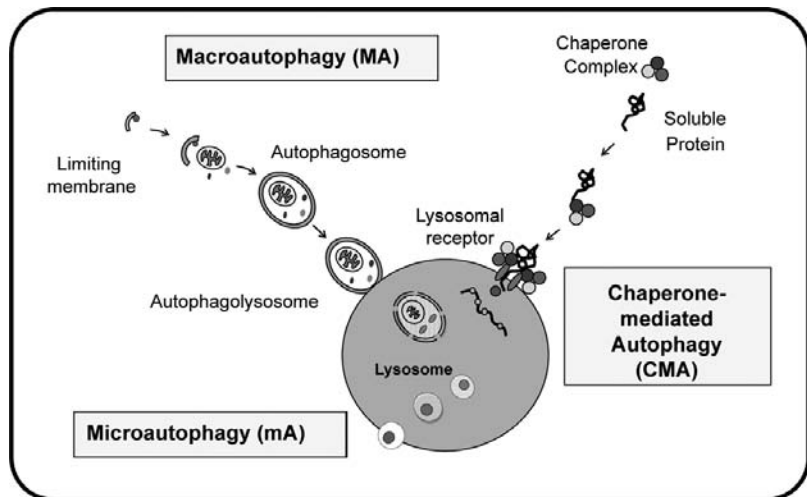


Figure 7-2. Schematic model of the most common types of autophagy in mammals. Three types of autophagy are quantitatively the most important in mammalian cells. Macroautophagy involves the sequestration of cytosolic components into a double membrane vesicle that then fuses with lysosomes to attain complete degradation of the cargo. In microautophagy, the lysosomes engulf whole cytosolic regions into small vesicles that pinch off from the lysosomal membrane and are degraded along with their cargo in the lumen. Chaperone-mediated autophagy is responsible for the degradation of specific cytosolic proteins that, after being recognized by a cytosolic chaperone complex, are delivered to lysosomes and cross the lysosomal membrane in a receptor-dependent manner.

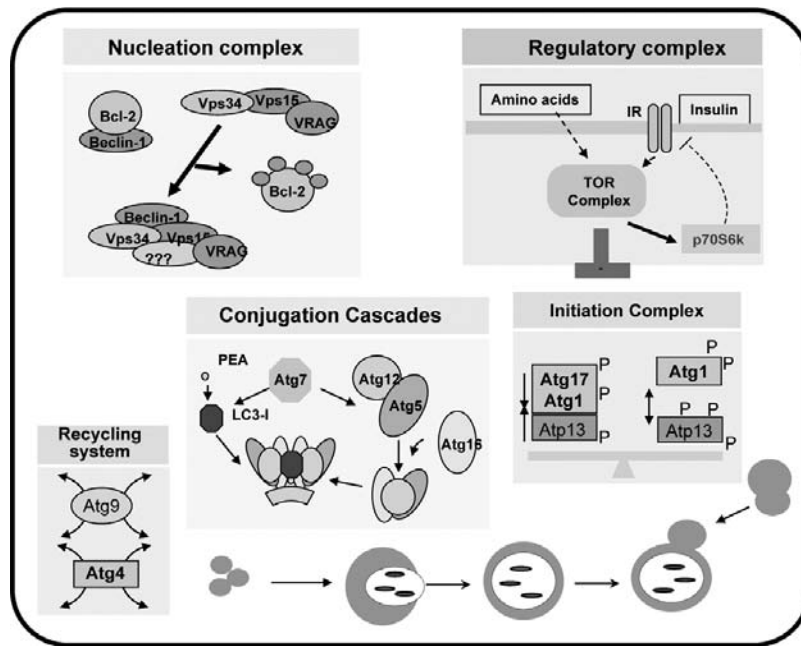


Figure 7-3. Molecular regulators and effectors of macroautophagy. The more than 30 genes that have been shown to participate in macroautophagy (ATG genes) can be subdivided into five main complexes: initiation complex, nucleation complex, conjugation cascades, recycling system, and the regulatory complex (see text for details).

existence in almost all cell types of basal macroautophagic activity essential for maintenance of cellular homeostasis.

Microautophagy, a type of autophagy still poorly characterized in mammals, has been proposed to be constitutively active and hence responsible for maintaining basal intracellular turnover. As in macroautophagy, complete regions of cytosol are degraded in lysosomes, but in this case, the lysosomal membrane itself invaginates or tubulates to trap the cargo (Figure 7-2). Microautophagy is well characterized in yeast, where the exit from rapamycin-induced growth arrest (EGO) complex, in conjunction with TOR, positively regulates this pathway by inducing deformation of the vacuole membrane (the equivalent to lysosomes in yeast).

Chaperone-mediated autophagy (CMA) is a type of autophagy described so far only in mammals, which is responsible for the selective degradation of a subset of cytosolic proteins in lysosomes. Substrate proteins all contain a pentapeptide motif (KFERQ-like motif) that is selectively recognized by the cytosolic chaperone hsc70, a constitutive member of the 70-kDa family of chaperones (Figure 7-2). The substrate/chaperone complex is targeted to lysosomes where they interact with a lysosomal receptor for this pathway, the lysosome-associated membrane protein type 2A (LAMP-2A). After unfolding, the substrate protein is translocated

into the lysosomal lumen assisted by a luminal chaperone. Some level of basal CMA activity is detectable in all cell types, but this type of autophagy is maximally activated under stress conditions.

Most of the studies on the relation of autophagy with cell death have focused on macroautophagy, which is also the main emphasis of this chapter. However, it is noteworthy to point out that blockage of CMA in cultured cells leads to activation of apoptosis on exposure to stressors that usually do not induce cell death if CMA is properly functioning. Although the mechanisms behind the activation of cell death under these conditions are unknown, it is interesting that well-known apoptotic effector proteins such as Apaf-1, Tp53, endonuclease G, caspase 3, Bim, Bcl-2, and Bcl-X_L all contain in their amino acid sequence CMA-targeting motifs that make them putative sub-

strates for CMA. Further investigation is necessary to determine whether or not selective degradation of apoptosis-related proteins via CMA could induce or prevent progression of apoptotic cell death under different conditions.

2.2. Physiologic functions of autophagy

Autophagy is an essential mechanism for cell defense in response to different types of stressors (Figure 7-4). Nutritional stress is the best characterized of these stressors, as it is well established that starvation induces activation of autophagy in many different organs in mammals and in unicellular organisms such as yeast. Activation of autophagy under these conditions is essential for cell survival. In fact, this dependence on autophagy for survival was used as a read out in the genetic screenings used to identify genes essential for autophagy in yeast, because autophagy-defective mutants are viable under normal growth conditions but die rapidly during starvation. Shortly after the identification of the first genes essential for autophagy in yeast (ATG genes) and their mammalian homologs, this critical role of autophagy during starvation was also confirmed in mammals. Thus mice deficient in essential autophagy genes die within 1 day after birth because activation of autophagy is required to survive during the

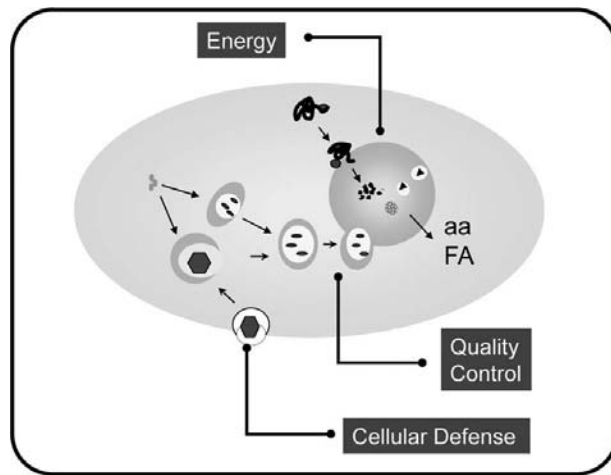


Figure 7-4. Physiologic functions of autophagy. Three of the many important functions of autophagy are to (1) become an alternative source of energy when nutrients are scarce by degrading proteins and lipid stores into amino acids (aa) and free fatty acids (FA); (2) contribute to quality control by eliminating any damaged protein and intracellular component; and (3) contribute to cellular defense against extracellular pathogens.

starvation that neonatal tissues face right after birth until nutrients can be restored through nursing.

Activation of autophagy during other types of stress responds, for the most part, to the need to eliminate intracellular components damaged by the different stressors (Figure 7-4). In other instances, activation of autophagy is used by cells as a mechanism of defense against pathogen invasion because autophagosomes have the capability to sequester and deliver the intruder to the lysosomal system for degradation.

Added to these well-characterized functions of autophagy as an inducible or stress-activated process, studies in the last few years have revealed a major role for autophagy in maintenance of cellular homeostasis under basal conditions. This basal autophagy has demonstrated to be an essential component of the cellular quality control mechanisms that prevent accumulation of cytotoxic proteins (aggregated and/or misfolded) and malfunctioning organelles (e.g., depolarized mitochondria, regions of the endoplasmic reticulum undergoing proteotoxic stress, nonfunctional peroxisomes). Autophagic removal of mitochondria is of particular relevance in the context of programmed cell death. Changes in the mitochondrial membrane potential and the resultant cytosolic release of mitochondrial components such as cytochrome c play a central role in the activation/maintenance of different cell death pathways. Consequently, a process such as macroautophagy that is able to sequester and eliminate the faulty

mitochondria could, at least in principle, prevent further engagement of downstream cell death effectors and hence avert cell death. In this respect, major effort is currently being dedicated to elucidate the mechanisms that mediate selective removal of altered mitochondria while preserving functional ones. Although still premature, recent studies support that active mitochondrial fusion and fission allow for the reorganization and sequestration of damaged mitochondrial components into daughter mitochondria that are segregated from the networking pool, recognized by the autophagosome integral components, and eliminated by autophagy.

2.3. Autophagy and human pathology

The improved methods to track autophagic activity in cellular and animal models, along with the capability now to modulate autophagy *in vivo* through genetic manipulations in ATG genes, has helped establish direct connections between autophagy malfunction and diverse pathologies, including neurodegenerative and metabolic diseases, infectious diseases, cancer, heart disease, and others.

Failure or inadequate autophagy has been proposed to underlie the pathogenesis of many late-onset neurodegenerative disorders caused by intracellular accumulation of pathogenic proteins that organize into toxic oligomers and higher order multimeric structures. As an essential component of the mechanisms for intracellular quality control, autophagy contributes to the continuous removal of these pathogenic proteins preventing cellular toxicity. In fact, upregulation of autophagy in several experimental models of proteotoxicity has proven effective in diminishing accumulation of protein aggregates and slowing down progression of disease. Defects in autophagy, often aggravated with age, have been extensively reported in the affected neurons in many of these disorders, suggesting that failure of this defensive mechanism is behind cellular loss and the subsequent onset of symptoms. Different components of the autophagic system seem to be direct targets of the pathogenic proteins. For example, mutations or post-translational modifications in α -synuclein, the protein that accumulates in the affected neurons in patients with Parkinson's disease, have been shown to directly interact with the CMA receptor at the lysosomal membrane, resulting in general CMA inhibition. Impaired macroautophagy has been reported in Huntington's and Alzheimer's diseases, although the mechanisms behind the autophagic failure remain, for the most part, unknown. In light of these

findings, autophagy is considered to be cytoprotective against neurodegeneration.

Recent studies in conditional knock-out mice with impaired autophagy in the pancreas have also revealed a critical role for basal macroautophagy in the homeostasis of beta cells, those responsible for insulin secretion. These findings, along with the failure of autophagy to remove altered secretory proteins such as insulin in diabetes patients, have strengthened the links between autophagy dysfunction and metabolic disorders.

Interestingly, in the four conditional mouse models with impaired autophagy in specific tissues developed so far (within neurons, cardiomyocytes, hepatocytes, and beta cells of the pancreas), the alterations in cellular homeostasis resulting from the autophagic failure inevitably lead to cellular degeneration and cell death by apoptosis.

The first connection between altered autophagy and disease was actually made with cancer, as impaired autophagy was identified as a common feature mammary and ovary cancers. Because the role of autophagy in carcinogenesis lies directly in the interplay between autophagy and cell death, we address it in more detail in the following sections.

3. AUTOPHAGY AND CELL DEATH

As described in the introduction, the involvement of autophagy in programmed cell death has been controversial. As expected from a stress-adaptation pathway that should promote cell survival, there is profuse evidence that disruption of autophagy or of the lysosomal system promotes cell death. Evidence in support of this prosurvival role of autophagy is discussed in the first part of this section (Figure 7-5, left). However, recent studies have also proposed that excessive or deregulated autophagy can lead to both apoptotic and non-apoptotic cellular death. This process is different from the described activation of apoptosis due to lysosomal enzyme leakage, which can initiate mitochondrial permeabilization and caspase activation. In the second part of this section, we discuss the arguments in sup-

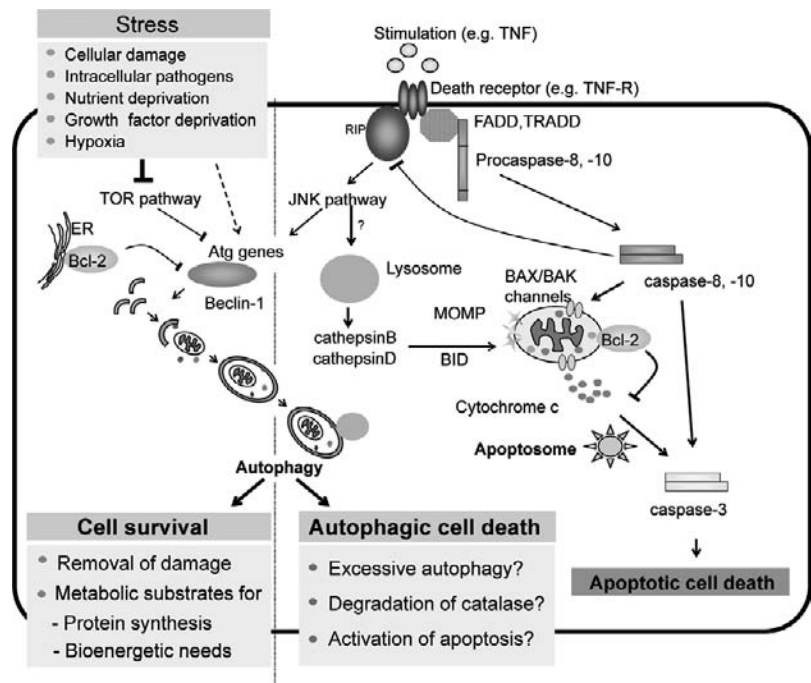


Figure 7-5. The dual role of autophagy as a cell defense and cell death mechanism. *Left:* Activation of autophagy protects cells against the damage caused by different types of stressors. *Right:* Caspases and autophagy are involved in complementary death pathways. Extracellular death ligands such as tumor necrosis factor (TNF) activate caspases that directly or through the involvement of mitochondria initiate apoptotic cell death. Death receptors also induce the release of active cathepsins from the lysosomal compartment. These cathepsins cleave Bid, which can then trigger cathepsin-mediated mitochondria outer membrane permeabilization (MOMP), inducing apoptotic cell death. If caspase-8 is inhibited, inhibition of RIP by caspase-8 is released and autophagic pathway is activated through JNK pathway and activation of Atg proteins, leading to autophagic cell death, although the precise mechanisms are not clear (see text). Unregulated exacerbation of autophagy or targeted removal of antiapoptotic factors may contribute to the detrimental effects of autophagic activation under these conditions.

port of and against autophagy as a cell death effector (Figure 7-5, right).

3.1. Autophagy as anti-cell death mechanism

Abundant evidence supports a cytoprotective function for autophagy in very diverse cellular settings and conditions (summarized in Table 7-1). As described in the previous section, genetic blockage of autophagy by deletion of essential autophagic genes in specific tissues in the mouse causes accumulation of poly-ubiquitinated protein aggregates, major alterations in cellular organelles, and cellular degeneration, thus arguing that a constitutive, low level of basal autophagy in normal tissues has an essential housekeeping function.

The prosurvival effect of autophagy encompasses the two major functions of this pathway, that of acting as an alternative source of energy and as a means for removal of altered cellular components (Figure 7-4). The

Table 7-1. Summary of work for and against autophagic cell death

Species	Treatment	Effect on autophagy	Tissue/ cell type	Role of autophagy
Autophagy as a prosurvival mechanism				
<i>Saccharomyces cerevisiae</i>	ATG gene mutants and starvation	Decrease		Adaptation to starvation
<i>Caenorhabditis elegans</i>	RNAi: unc-51, bec-1, atg7, atg8, atg16, bec-1, atg8, atg18	Decrease		Early/larval development
<i>Drosophila</i>	RNAi: atg1, atg3	Decrease		Larval/pupal development
Mouse	RNAi: Atg5, Atg7	Decrease	Brain	Accumulation of polyubiquitylated proteins/degeneration
	Atg5 (RNAi)	Decrease	T and B cells (peritoneum)	T-cell survival/proliferation B-cell development
	Interleukin-3 withdrawal	Increase	Bax ^{-/-} Bak ^{-/-} cell	Maintenance of cellular ATP Degradation of glucose transporter
	Over-expressed bec-1 virus infection	Decrease	brain	Protection against virus-induced encephalitis
Human	Starvation	Increase	HeLa cells	Adaptation to starvation
	Oxidative stress	Increase	Several	Damaged mitochondria removal
	mTOR inhibitors	Increase	Several	Cell growth/proliferation
Mouse	RNAi: Atg5, Beclin1 3-methyladenin	Decrease	Bax ^{-/-} Bak ^{-/-} MEF	Etoposide/staurosporine-induced cell death
	Chloroquine	Decrease	Neurons	Autophagic plus partially apoptotic cell death
	Fibroblast growth factor (withdrawal)	Increase	Neuronal	Autophagic cell death
Rat	Nerve growth factor (withdrawal)	Increase	Neurons	Autophagic/apoptotic death
	Serum deprivation	Increase	Pheochromocytoma	Autophagic cell death cathepsin D B
	N-meth-D-aspartate	Increase	Neurons	Autophagic cell death
Human	RNAi: Atg7, Beclin1	Decrease	Fibroblasts monocytes	z-VAD-induced cell death
	RNA: Atg5, 10, 12 Beclin-1, Vps34	Decrease	HeLa cells	Apoptotic cell death
	RNAi:LAMP2 pH Neutralizers	Decrease	HeLa cells	Apoptotic cell death
	Bafilomycin	Decrease	Glioblastoma	Toxic-induced cell death
	3-MA Antiestrogen	Decrease Increase	Breast cancer	Irradiation or tamoxifen-induced cell death
	ceramide	Increase	Glioma cells	Autophagic and apoptotic cell death

capability of autophagy to maintain a positive cellular energy balance is particularly important during nutrient deficiency. Degradation of proteins and even intracellular lipid storages by autophagosomes generates amino acids and free fatty acids that can be used for de novo protein synthesis to support other metabolic pathways such as tricarboxylic acid cycle or to fuel mitochondrial adenosine triphosphate energy production through β -oxidation. This function of autophagy in recycling underlies the ability of this pathway to sustain life during starvation.

This role of autophagy in maintaining cellular bioenergetics has recently proven essential in conditions other than starvation. Thus autophagy is also activated in response to growth factor deprivation or during hypoxia. The rapid degradation of the glucose transporter that follows growth factor withdrawal leaves cells in a compromised energetic balance, but this is prevented by activation of autophagy, which can maintain intracellular ATP levels compatible with cell survival for several weeks.

As described in the previous section, the ability of autophagy to remove defective intracellular components also has a protective effect against cell death. Removal of toxic forms of proteins by autophagy is essential for neuronal survival in various neurodegenerative disorders. Regarding organelles, mitochondria have been the organelle most extensively analyzed given their critical role in cell death pathway. Both the cell death observed on blockage of basal or inducible autophagy depends on mitochondrial outer membrane permeabilization and subsequent caspase activation. However, recent studies support that timely removal of other compromised organelles by autophagy is also essential in preventing cell death. Thus activation of autophagy is often part of the response to endoplasmic reticulum (ER) stress, and failure to activate autophagy under these conditions precipitates cell death both in yeast and in mammalian cells. The high capability of the autophagic systems may be advantageous in certain conditions for the degradation of the compromised ER when compared with the proteasome-mediated degradation of misfolded ER proteins.

Although activation of autophagy has been observed in multiple cellular conditions and in response to numerous stressors, the most convincing evidence of the prosurvival role of this pathway has resulted from genetic studies. Blockage of autophagosome formation in many of those conditions precipitates cell death, supporting thus that the observed activation of autophagy is a cellular survival strategy.

3.2. Autophagy as a cell death mechanism

Autophagic cell death has been historically defined by morphological criteria, namely presence of structures compatible with autophagosomes in a dying cell. However, in recent years it has become clear that the mere presence of autophagosomes is insufficient to distinguish cell death with autophagy from cell death by apoptosis. In that respect, the most convincing way to show active participation of autophagy in cell death in a given situation is to demonstrate that blockage of autophagy by manipulation of essential autophagic genes prevents cell death. In fact, multiple reports have now shown decreased apoptosis on inhibition of autophagy under different conditions. For example, silencing of Atg7 or Beclin-1 inhibits the autophagic cell death of L929 cells induced by the pancaspase inhibitor Z-VAD-fmk (N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone). Similarly, embryonic fibroblasts derived from mice that lack the function of the Bcl-2 family member (Bax^{-/-}Bak^{-/-} MEF) are resistant to apoptosis (by treatment with etoposide) but die by autophagic cell death that requires Atg5 and Atg6 function.

However, as a note of caution, a beneficial prosurvival effect of blockage of autophagy can be misleading under certain conditions. Thus, for example, during autophagic stress resulting from the inability of lysosomes to clear autophagosomes, a decrease in autophagosome formation may give the cells a temporary “break.” Massive accumulation of autophagosomes inside cells, as the one observed in some neurodegenerative disorders or in some vacuolar myopathies, results in major alterations in cellular trafficking, energetic dysbalance, and often compromised stability of the autophagosome, with the consequent cytosolic leakage of undegraded toxic cellular products. Under these conditions, knockdown of the genes involved in autophagosome formation will reduce the total autophagosome content and may give time to the lysosomal system to accommodate the clearance of the remaining autophagosomes, with the consequent beneficial effect for the cell. Often, in these circumstances, the original upregulation of the autophagic system was part of the cellular defense against intra- or extracellular stressors, and consequently, it should not be classified as cellular death by autophagy, because it is the failure to perform a complete degradative autophagy that leads to compromised cell viability. Different authors have proposed the more appropriate term *cell death with autophagy* to refer to these conditions. One additional variation on this theme has been

described in the case of some viral and bacterial infections that use the autophagic machinery for their own survival, assembly, and proliferation. Preventing formation of autophagosomes under these conditions also has a beneficial effect in the infected cells by limiting the ability of the pathogen to survive and colonize the cell. As in the previous example, autophagy cannot be considered an active effector in cell death under these conditions, because it is the failure of the autophagic system to eliminate the pathogen that leads to colonization and cell death.

Despite these notes of caution when defining autophagic cell death, studies in different invertebrate systems, such as the fat body of the fly, have shown evidence in support of an active role of autophagy in cell death. In most of these instances, cell death is related to tissue differentiation, remodeling or embryogenesis.

Exacerbation of the autophagic pathway has also been shown to lead to cellular death, at least in cultured cells and invertebrates. The mechanisms linking excessive autophagy with cell death are still not clear, but the most intuitive explanation is that an imbalance in cell metabolism, in which autophagic cellular consumption exceeds the cellular capacity for synthesis, exhausts the cellular resources and eventually promotes cell death.

3.3. Molecular players of the autophagy–cell death cross-talk

The molecular mechanisms of autophagic cell death are, for the most part, still unknown. One of the first components proposed to regulate the cross-talk between autophagy and apoptosis has been the protein pair Beclin-1/Bcl-2. The initiation complex Beclin-1/Vps34 is negatively regulated by Bcl-2 in a nutrient-dependent manner. Bcl-2 family proteins are key regulators of apoptosis that are represented by anti-apoptotic proteins, such as Bcl-2, and proapoptotic proteins Bax and Bak, which regulate the efflux of proapoptotic molecules from mitochondria and possibly other organelles. Deficiency in Bax and Bak or expression of Bcl-2 results in marked resistance to many apoptotic stimuli. Binding of Bcl-2 to Beclin-1 prevents activation of autophagy, whereas knockdown of Bcl-2 or over-expression of Beclin-1 mutants unable to bind Bcl-2 results in unregulated massive autophagy and cell death. Thus the anti-oncogenic role of Bcl-2 may result not only from its ability to block apoptosis, but also from its ability to prevent unregulated (excessive) autophagy. The detrimental role of excessive autophagy has been recently confirmed in studies with *Caenorhabditis elegans*.

The autophagic protein Ser/Thr kinase Atg1 also appears to act as a convergence point for signals linking autophagic and apoptotic cell death. In *Drosophila*, over-expression of Atg1 is sufficient to induce high levels of autophagy that lead to caspase-dependent apoptotic cell death. The stimulatory effect of Atg1 on autophagy seems to depend on its ability to inhibit TOR signaling, although it is also possible that part of the autophagic upregulation is distinct from the TOR control. In fact, Vps34 has been recently shown to promote autophagy but not TOR signaling in *Drosophila*, although so far, in most mammalian cell types analyzed, the effect of Vps34 seems to be dependent on changes in TOR signaling. This may reflect a fundamental difference in signaling mechanisms between the fly and mammalian systems.

A second line of thought in the identification of the molecular regulators of autophagic cell death supports that excessive autophagy may degrade cytoprotective effectors. For example, removal of catalase by Jun kinase-regulated autophagy leads to cellular accumulation of reactive oxygen species (ROS) and lipid peroxidation products and eventually precipitates cell death. Finally, another appealing possibility is that particular autophagic gene products, when expressed at high levels or after post-translational modification, directly activate apoptosis in a manner independent of their effect on autophagy.

4. AUTOPHAGY, CELLULAR DEATH, AND CANCER

Of the various human disorders for which a connection with autophagy has been established, cancer is probably the one for which the relationship between autophagy and cell death has been most extensively explored. In this context, a dual anti-oncogenic and pro-oncogenic role of autophagy has also been described (Figure 7-6).

Downregulation of autophagy is a common feature of many cancer cells and has been shown to be necessary to maintain their oncogenic potential. In fact, expression of endogenous Beclin-1 protein is frequently low in human breast epithelial carcinoma cell lines, and restoration of normal levels of this protein or activation of autophagy by other means diminishes the tumorigenic capability of these cancer cells. Different mechanisms have been proposed to explain the anti-oncogenic effect of autophagy (summarized in Figure 7-6). For example, a switch from a catabolic to an anabolic status when autophagy is reduced will favor cellular growth and division and tumor progression. In addition, reduced autophagy could also stimulate oncogenesis by favoring a proinflammatory

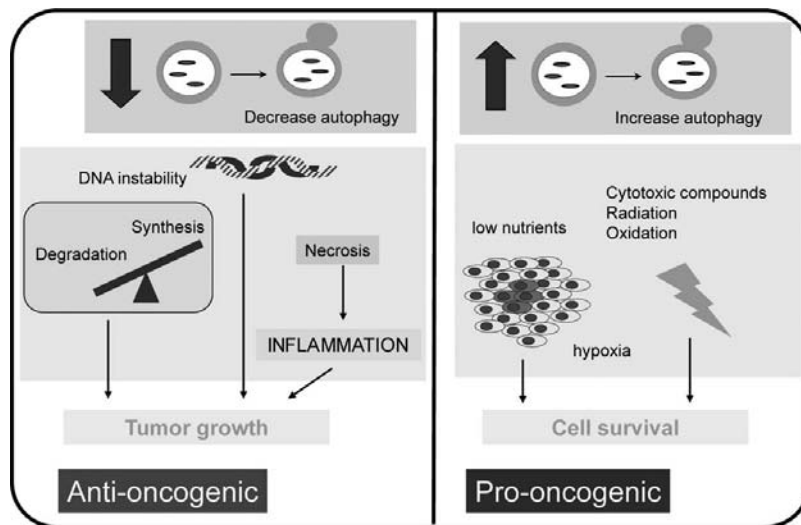


Figure 7-6. Paradoxical function of autophagy in cancer biology. *Left:* Anti-oncogenic role of autophagy. Reduced autophagy favors cell proliferation and DNA instability and may facilitate progression of necrosis. The inflammation associated with necrosis creates a niche that further stimulates growth of cancer cells. *Right:* Pro-oncogenic role of autophagy. Activation of autophagy is necessary for survival of cells in the center of poorly vascularized tumors and as defense against damage induced by anti-oncogenic treatments.

environment known to increase tumor growth rate. Thus, only when autophagy is repressed, tumor cells that cannot die by apoptosis on exposure to metabolic stress die by necrosis, a process known to exacerbate local inflammation. Reduced autophagy may also promote cancer by increasing genomic instability, leading to oncogenic activation and tumor progression. Indeed, immortalized mouse epithelial cells with impaired autophagy display increased DNA damage, centrosome abnormalities, structural chromosomal abnormalities, and gene amplification, conditions all associated with increased tumorigenicity. However, because all these studies have been performed with cells engineered to have concurrent defects in apoptosis (e.g., p53 and Rb inactivation, Bcl-2 overexpression), it is not yet possible to conclude that autophagy limits genome damage in normal cells and thereby plays a role in preventing tumor initiation.

Another plausible explanation for the anti-oncogenic effect of autophagy is that this catabolic pathway plays a direct role in negative growth control, perhaps by degrading specific organelles or proteins essential for cell growth regulation. In support of this theory, the previously mentioned enforced Beclin-1 expression slows the proliferation of tumor cell lines (without affecting cell death) and causes a decrease in expression of cyclin E and phosphorylated Rb. In *Drosophila*, overexpression of Atg1, which causes the hyperactivation of autophagy, directly inhibits cell growth and induces cell death. Different components of the Beclin-1/

class III (PI [3]) kinase complex, such as ultraviolet radiation resistance-associated gene (UVRAG) and Bif-1, have been proposed to modulate the regulatory effect of Beclin-1 in cellular growth and tumorigenesis. The regulation of autophagy by signaling pathways overlaps with the control of cell growth, proliferation, cell survival, and death. Several tumor suppressor genes (phosphatase and tensin homolog [PTEN] and p53) involved in the TOR signaling network have been shown to stimulate autophagy. In contrast, the oncoproteins involved in this network have the opposite effect. Interestingly, and in accordance with the ability of different types of cancer cells to turn autophagy on and off depending on the tumoral stage, particular tumor suppressors such as p53 have also shown to have dual effect on

autophagy. Thus, in contrast to the stimulatory effect on autophagy of nuclear p53, the cytosolic form of this protein has been recently shown to have a tonic inhibitory effect on autophagy in human, mouse, and nematode cells. The role of p53 in cancer has gained thus an extra level of complexity, as it not only inhibits the antiapoptotic effect of Bcl-2 homologs and activates Bax and Bak, which promote apoptosis, but it also modulates autophagy. Although the precise molecular mechanism by which p53 inhibits autophagy remains under investigation, these results provide evidence of a key signaling pathway that links autophagy to the cancer-associated dysregulation of p53.

The constitutive low levels of autophagy often observed in a growing tumor do not reflect, however, a complete inability of cancer cell to perform autophagy. As in almost all cell types, upregulation of autophagy has been also observed in cancer cells faced with a variety of stresses, such as oxidative damage, hypoxia, cytotoxic compounds, blockage of the proteasome, ER stress, or mitogen-activated protein kinase signaling. Using cell lines deficient in apoptosis, multiple investigators have reported that activation of autophagy in response to these stressors allowed tumor cells to survive. For example, in Bak^{-/-}Bax^{-/-} cells, autophagy serves to sustain cells during interleukin-3 withdrawal. Furthermore, autophagy is essential for cancer cells to survive the hypoxia and poor nutritional conditions of the center of large solid tumors before angiogenesis occurs. The direct mechanisms by which these

different stressors induce autophagy are poorly understood. Accumulation of ROS, produced under many cellular responses to stress, can directly activate autophagy by inactivation of the cysteine protease Atg4. Blockage of this protease leads to accumulation of the Atg8-phosphoethanolamine precursor required for the formation of autophagosomes. Current research efforts are focused on identifying this type of connections between stress and autophagy as they could become perfect targets of therapeutic approaches aimed to increase the susceptibility of cancer cells to anti-oncogenic treatments.

In summary, autophagy can have opposite functions (pro- or anti-oncogenic) in different steps of tumorigenesis and depending on the environmental conditions that surround the tumor.

5. CONCLUDING REMARKS AND PENDING QUESTIONS

If we have learned anything through recent studies of the role of autophagy in cell death, it is that the answer is never absolute. The better understanding of the autophagic process and its interplay with apoptosis and other forms of cell death is helping to reconcile the initially conflicting views of autophagy as a prosurvival or cell death mechanism. For example, the better definition of autophagy as the process leading to not only engulfment, but also to complete degradation of the sequestered cargo has replaced autophagy by “inefficient autophagy” as a cause of cell death, regaining thus a prosurvival role for autophagy in some of these conditions. However, even when the most strict criteria of what is understood by autophagy are applied, there are still clear conditions in which autophagy becomes an effector of cell death. In most of these cases, except for those related to embryogenesis and tissue remodeling, it is an excess in the rates of autophagy that leads to cell death, likely through consumption of cellular components essential for survival.

One aspect that has clearly increased the complexity of the role of autophagy in cell death is the fact that different cells exposed to the same cell death stimuli respond differently, and by the same token, the same cell type also responds in a different manner when exposed to different cell death stimuli. Are there specific autophagic responses for individual death stimuli? The answer to this question may become clear as we gain a better understanding about other types of autophagy and the cross-talk mechanisms among autophagic pathways and cellular systems. For example, in the same way that cells with impaired CMA can survive nutritional stress through compensatory activation of

macroautophagy, activation of CMA often detected as compensatory mechanism for impaired macroautophagy could also be beneficial in response to particular cell death stimuli. Thus recent studies have shown that exposure of mouse fibroblasts with compromised macroautophagy to Fas/tumor necrosis factor- α induces caspase-dependent apoptosis, whereas these cells become resistant to death from menadione and ultraviolet light because of upregulation of CMA.

Also unclear is the value of autophagic sequestration versus lysosomal degradation in the prosurvival effect of autophagy. Intuitively, sequestration – for example, of a leaky mitochondrion – even if its degradation cannot be completed, should be better than leaving the organelle free in the cytosol. However, the consequences of the accumulation of undegraded autophagic vacuoles in the cytosol (autophagic stress) should not be underestimated. Consequently, alterations in both the formation of autophagosomes or the degradation of autophagic vacuoles can lead to cell death, although the mechanisms are probably different. These findings force reevaluation of those interventions aimed to enhance cell survival by only upregulating autophagosome formation. Simultaneous upregulation of autophagosome formation and clearance should be the gold standard of any manipulation on the autophagic pathway with therapeutic purposes.

A current limitation of some of the current studies on the role of autophagy in cell death is that whereas we count on efficient genetic methods to inhibit autophagy through downregulation of autophagy genes, our means to upregulate autophagy, at least in mammals, are still very limited. In particular, good pharmacological regulators are unavailable, as all the compounds available today (such as mammalian TOR [mTOR], histone deacetylase [HDAC], or Akt inhibitors) also control other important processes in the cell. There is thus a pressing need to develop compounds that target selectively Atg proteins, which could then be used to directly address the role of different types of autophagy in cellular survival and death in response to particular stimuli.

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8

Cell Death in Response to Genotoxic Stress and DNA Damage

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Cells are subjected to multiple types of stress throughout their life cycle, including starvation, infection, and physical and chemical agents. Stressors cause transient and permanent damage. Transient damage is reflected at the level of the protein or RNA and is largely associated with the generation of reactive oxygen radicals, which directly or indirectly impact translation, folding, or conformation of proteins. In contrast to transient damage, which is expected to be cleared by existing cellular machinery that allows recognition and removal of damaged proteins, permanent damage is primarily reflected at the level of the DNA, although it could also result from damaged proteins that fail to support proper repair or cell duplication. DNA-damaging agents induce a variety of modifications that may result in improper chromosomal duplication, recombination between chromosomes, gene mutations, or gene amplification, which may result in malignant transformations if not properly repaired. Damage is generated by both endogenous and exogenous sources: endogenous (spontaneous) damage is caused by agents within the cell itself (i.e., the products of normal cellular metabolism, replication, mitosis), whereas exogenous sources include ultraviolet (UV) light, ionizing radiation (IR), and environmental genotoxins (e.g., alkylating compounds, polycyclic aromatic hydrocarbons, biphenyls, and heterocyclic amines). Most cytotoxic anticancer drugs react either directly or indirectly (through reactive metabolites) with DNA or by blocking DNA-metabolizing functions, such as DNA polymerases or topoisomerases.

To cope with DNA damage, two cellular strategies have evolved in multicellular organisms: (1) DNA damage is repaired or tolerated; or (2) cells harboring DNA damage are removed from the population by apoptosis or other forms of cell death. This contrasts with unicellular organisms, in which the best way to ensure

survival is simply to repair any damaged DNA. Metazoans, however, are better served by having alternate strategies that enable a means of destroying irreparably damaged cells. For example, extensive damage of the DNA is likely to result in multiple genetic mutations that cannot be tolerated, therefore triggering a programmed cell death response. As a result of this flexibility, repair, growth arrest, and apoptosis are all possible cellular responses to genotoxic stress in metazoans, with the choice dependent on cell type, location, environment, and extent of damage.

1. TYPES OF DNA DAMAGE AND REPAIR SYSTEMS

Endogenous DNA damage occurs at a higher frequency than exogenous injury. Yet in developed countries, accidental or involuntary exposures to exogenous genotoxic factors contribute to 75% to 80% of cancer cases. Notably, both endogenous and exogenous sources induce similar types of DNA lesions such as modified bases, abasic sites, single-strand breaks, helix-distorting adducts, intra- and interstrand cross-links, and double-strand breaks (DSBs). If not repaired, these lesions may result in base transitions, transversions, frameshift mutations, or chromosomal aberrations.

To recognize and remove damaged bases or more complex DNA lesions, a cell has access to at least five mechanisms: (1) base excision repair, (2) nucleotide excision repair, (3) mismatch repair, (4) direct reversal of damage, and (5) recombinational repair. Principal agents that cause DNA damage, the resulting lesions, and DNA repair mechanisms are outlined in [Table 8-1](#).

Base excision repair, nucleotide excision repair, and mismatch repair remove modified bases, mismatches, and bulky adducts by removing the substrate base, forming a single-strand break gap at the excision site and

Table 8-1. DNA lesions and repair pathways

Source of damage	Type of damage	DNA repair pathway
Ultraviolet radiation	Cyclobutane pyrimidine dimers (TT, TC, CT, or CC)	NER
Ultraviolet radiation	Bulky or helix-distorting DNA lesions	NER
Ultraviolet radiation	(6-4) photoproducts	NER
Ionizing radiation	Double-strand breaks	HR/NHEJ
Ionizing radiation	Single-strand breaks	BER
Ionizing radiation	Oxidative base damage	BER
Mitomycin	Interstrand crosslinks	RR
Cisplatin	Intrastrand crosslink	NER
Alkylating agents	O ⁶ -alkylguanine	DRD
Alkylating agents, spontaneous hydrolysis	Non-helix-distorting base modifications, abasic sites	BER
Aldehydes	DNA adducts	NER
ROS	Oxidative base damage	BER
ROS	Cyclopurines (A or G) making bulky lesions	NER
Replication errors	Mismatches, small insertions or deletions	MMR
Collapsed replication forks	One-ended double-strand breaks	HR

Note: BER, base excision repair; DRD, direct reversal of damage; HR, homologous recombination; MMR, mismatch repair; NER, nucleotide excision repair; NHEJ, nonhomologous end joining; RR, recombinational repair; ROS, reactive oxygen species.

finally reestablishing the original DNA content using pathway-specific polymerases and ligases. Base excision repair acts during all cell cycle stages and is often responsible for correcting damages that arise spontaneously due to the inherent instability of DNA or due to exposure to intercalating (i.e., anticancer) agents and environmental mutagens that generate free radicals. Nucleotide excision repair largely acts independently of the cell cycle to remove bulky DNA adducts, such as UV-induced cyclobutane pyrimidine dimers, DNA cross-links, and certain oxidative base modifications. Mismatch repair acts to remove not only mismatches, but also small insertions or deletions that arise as replication errors or that arise during recombination.

Direct reversal of damage is a highly specialized repair mechanism. In humans, only the MGMT (O⁶-methylguanine-DNA methyltransferase) protein is known to function by this repair mechanism and irreversibly accepts the methyl group directly from the modified base.

Recombinational repair is required to repair DSB and is thus especially important in response to IR. DSBs are among the most harmful of lesions because they affect both strands of the double helix, meaning that one strand of DNA cannot act as a template for the repair of the other. There are two forms of DSBs: (1) two-ended breaks, generated primarily by direct attack on DNA by a physical or chemical mutagen such as IR, and

(2) one-ended breaks, created when the replication fork collides into an unrepaired DNA single-strand break. One-ended breaks appear to be resolved strictly by classical homologous recombination. Two-ended breaks are repaired by three major repair mechanisms: (1) homologous recombination, (2) single-strand annealing, and (3) nonhomologous end-joining. Homologous recombination usually takes place after DNA replication (i.e., during S or G2 phase of the cell cycle) and is largely error-free. An undamaged, homologous molecule such as a sister chromatid provides the repair template. On the other hand, single-strand annealing is an error-prone repair system. Homologous sequences (usually repetitive elements) on either side of the DSB are aligned followed by the deletion of the intermediate noncomplementary sequence. Nonhomologous end joining (NHEJ) is the major DSB repair pathway that takes place during the G1 phase of the cell cycle. This pathway is prone to errors because it fuses together two ends of a DSB.

2. DNA DAMAGE RESPONSE

An important question in cell biology is how the cell detects DNA damage. The cellular response to genotoxic stress can be envisioned as a highly conserved signal transduction cascade: the DNA damage response (DDR) (Figure 8-1). Sensor proteins are the first to detect DNA damage and replication stress. They then

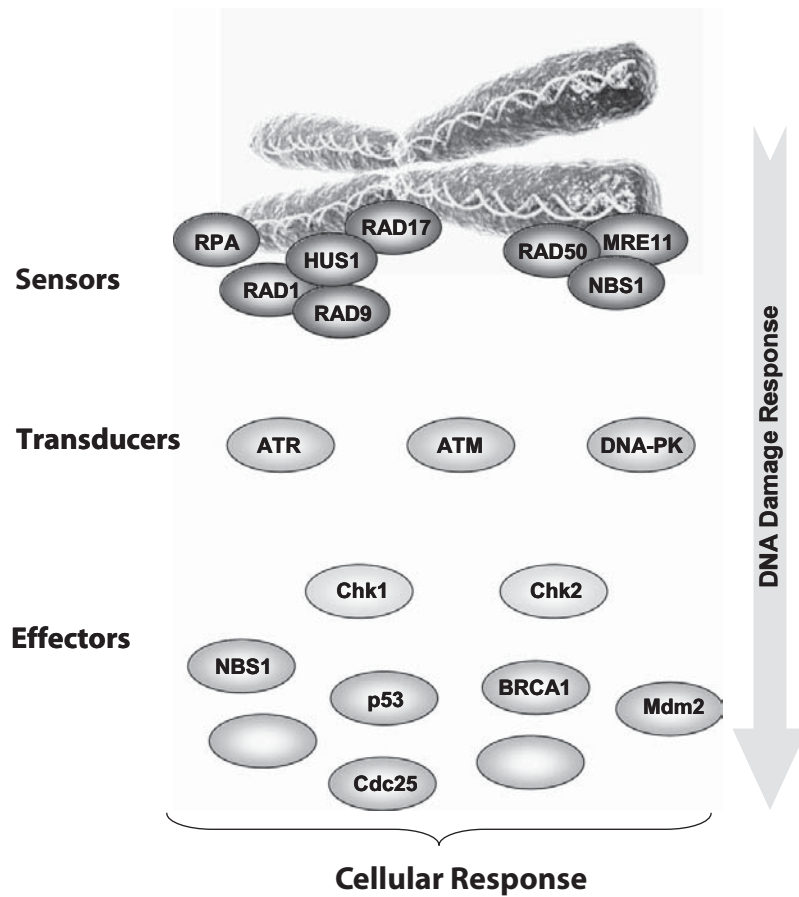


Figure 8-1. A general representation of the DNA damage response. DNA damage is sensed by sensor proteins. This signal is transduced by transducers to effector proteins, which mediate the cellular responses to DNA damage.

transmit a signal to transducer proteins, which are composed primarily of protein kinases that are activated through phosphorylation. Eventually, the signal is conveyed to numerous effector proteins that execute various cellular functions, including DNA repair, cell cycle checkpoints, cellular senescence, and apoptosis.

2.1. Sensors

Clearly, sensing the lesion is the first essential step in the DDR. On DNA damage, different multiprotein complexes, the composition of which is determined by the specific type of damage, bind the lesion on the DNA. For instance, the MRN complex, composed of 3'–5' exonuclease MRE11A, Rad50 ATPase, and a regulatory protein defective in Nijmegen breakage syndrome (NBS1), is generally thought to play an early role in detecting and processing DSBs. A different DNA damage response pathway is activated in response to replication fork stalling and single-strand breaks. When DNA polymerases stall, helicases continue unwinding DNA ahead

of the replication fork, which generates single-strand DNA that is sensed and bound by the single-strand binding protein complex replication protein A.

The multiprotein complexes rapidly expand to form nuclear foci (DNA damage heterochromatin foci). Highly dynamic and massive (giga-Dalton sized), the foci contain hundreds of individual DNA repair and checkpoint proteins, modified chromatin, and damaged DNA. Foci, a punctuate or speckle seen on immunostaining with antibodies, is a hallmark of the DNA damage response, and its main function is to cluster DNA damage response proteins at the damaged sites. Foci are often seen within minutes after DNA damage and remain visible up to 24 hours after the damage, long after it is repaired.

2.2. Transducers

Various kinases of the phosphoinositide-3-kinase-related protein kinase (PIKK) family, including ataxia-telangiectasia mutated (ATM) kinase (the gene altered in this recessive human genomic instability syndrome), ATM- and Rad3-related (ATR) kinase, and DNA-dependent protein kinase (DNA-PK), constitute the primary transducers of the DNA damage response. Within minutes of the DSB formation, ATM is recruited to the foci and activated. Active phosphorylated ATM remains stable for many hours. Unlike ATM, the ATR gene and its canonical substrate, Chk1, are essential in mice, underscoring their important role in normal cell growth. The ATR pathway is normally activated by stalled replication forks during DNA replication and thus plays an essential role in maintaining genome integrity during S phase. UV light, single-strand DNA, and presumably all chemical agents that give rise to stalled DNA replication forks also strongly activate the ATR pathway.

ATR is recruited by the single-strand DNA-RPA complex that also recruits and activates Rad17 and the proliferating cell nuclear antigen (PCNA)-related 911 (Rad9-Rad1-Hus1) complex. ATR phosphorylation of Rad17 and 911 is important for downstream signaling. It is not yet clear how ATR is activated when recruited to single-strand DNA lesions, although both the 911 complex and TopBP1, another protein in the complex, have been



Figure 8-2. In response to DNA damage, the cell activates checkpoint arrest to facilitate repair of damage. On successful repair, the cell cycle resumes. If DNA damage is too severe or cannot be repaired, the cell activates senescence or apoptosis.

shown to stimulate ATR kinase activity. ATM and DNA-PK activity is preferentially triggered by DSBs induced by IR. ATM exists as inactive dimers that, when recruited by the Mre11, Rad50, and Nbs1 (MRE) complex to the foci, become activated on multiple residues by dissociation and autophosphorylation. The MRN complex is also a substrate of ATM whose phosphorylation is important for downstream signaling.

2.3. Effectors

ATM and ATR regulate cell cycle progression and arrest, DNA repair systems, cellular senescence, and apoptosis by activating a host of effector proteins (Figure 8-2). After the DNA damage response is activated, phosphorylation (mediated by ATM and ATR) and other post-transcriptional modifications (notably, ubiquitination and methylation) induce chromatin remodeling and further recruitment of proteins such as p53, Mdm2, BRCA1, FANCD2, and NBS1 to the foci. The identity of the proteins that regulate DNA repair and the damage signal depends on the nature of the damage and during what phase of the cell cycle that the damage has taken place.

Several adaptor proteins, including 53BP1, BRCA1, MDC1, and claspin, organize the synchronized recruitment of DNA damage response proteins, as well as the function of downstream kinases such as checkpoint-1 (Chk1) and checkpoint-2 (Chk2). ATR induces Chk1 phosphorylation at Ser317 and Ser345, which is thought to facilitate Chk1 function. ATM induces Chk2 phosphorylation at Thr68, which triggers Chk2 activation through homodimerization and autophosphorylation at Thr383 and Thr387. To date, more than 700 proteins have been identified as candidate substrates phosphorylated by ATM and ATR in response to IR or UV. The studies revealed proteins involved in DNA replication and various DNA repair mechanisms, highlighting the critical role of the DDR in controlling genomic stability. Interestingly, proteins belonging to pathways not directly implicated in the DDR, such as insulin signaling, RNA splicing, nonsense-mediated RNA decay, the spindle checkpoint, mitotic spindle and kinetochore proteins,

tumor suppressors, and chromatin remodeling, were also identified among ATM/ATR substrates.

3. INTEGRATION OF ATM AND ATR PATHWAYS

Because ATM and ATR respond to very different stimuli, they have been considered analogous components of independent and parallel pathways but with distinct inputs and outputs. However, multiple genomic insults eventually activate both kinases, which ultimately trigger Chk1 and Chk2 activation. ATR responds robustly to DSBs, and the response is ATM-dependent. The recruitment of ATR to the location of DSBs by ATM appears to be an indirect effect because ATM triggers the formation of a DNA-protein structure that provides a strong stimulus for ATR signaling. Similarly, UV and hydroxyurea, both potent activators of ATR signaling, also activate ATM and, importantly, this activation is ATR-dependent. Collectively, these studies demonstrate that ATM and ATR function as an integrated molecular circuit to process diverse signals. Consequently, they effectively link the DNA replication apparatus with DDR pathways. Supportive of this cross-talk between ATM and ATR is that both phosphorylate the same consensus sequence on their substrates.

4. CHROMATIN MODIFICATIONS

Efficient repair of DNA damage is challenged by the physical state of genomic DNA, which is highly compacted and condensed within the chromatin. The most basic component of chromatin, the nucleosome, consists of 147 bp of DNA wrapped around a histone octamer (two copies each of histones H2A, H2B, H3, and H4). To manipulate the chromatin-packaged state of DNA, specialized mechanisms have evolved, including covalent histone modifications (phosphorylation, methylation, acetylation, ubiquitination, sumoylation, and adenosine diphosphate ribosylation), ATP-dependent chromatin remodeling, and histone variant incorporation.

DNA damage triggers alterations in chromatin structure, including dynamic and specific post-translational covalent modifications of histone proteins that are thought to play critical roles in surveillance, detection, and repair. The first damage-specific histone modification identified was phosphorylation of H2A S129 (H2AX S139 in mammalian cells) by ATM/ATR and DNA-PK. H2AX phosphorylation occurs immediately after a DSB

and has become a standard marker for such damage. Although unnecessary to promote the initial steps of the repair process, this modification is needed to concentrate repair machineries along the DNA lesions and to recruit chromatin modifiers such as complexes INO80, Swr1, and NuA4, which relaxes the chromatin structure surrounding the DNA lesion.

PP2A dephosphorylation of H2AX has been recently shown to be significant in turning off the damage response. Other H2A modifications have been identified as signals for general damage or stress, whereas others play roles in distinct repair pathways. Specifically, H2A phosphorylation of S122 and S129 is required to repair DSBs either by homologous recombination or NHEJ pathways. Other residues have even more specific roles: T126 is important for homologous recombination but dispensable for NHEJ, whereas S2 and K127 are critical for NHEJ but have no role in homologous recombination. These data indicate that both the type of damage and selected repair pathway are marked by specific H2A modifications, creating a unique histone code for each type of damage and repair.

Another covalent histone modification implicated in the DNA damage response is the methylation of histone H3 at lysine 79 (H3K79me) by the histone methyltransferase Dot1. Unlike γ H2AX, DNA damage does not induce H3K79me but is constitutively present on chromatin. Otherwise, DNA damage would increase the accessibility of methylated H3K79me, allowing 53BP1 to instead act during the early sensing step. Similarly, H4K20 plays an analogous role in recruiting Crb2.

Histone acetylation not only functions in protein recruitment, but also acts to relax the chromatin structure and therefore facilitates access of DSB repair proteins such as 53BP1, BRCA1, and Rad51 to the lesion. The acetylation status of histones is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), and some of these HATs and HDACs are recruited to the lesion.

In addition to covalent histone modifications mentioned previously, chromatin is directly manipulated by adenosine triphosphate (ATP)-dependent chromatin remodeling complexes. These complexes use energy from ATP hydrolysis to facilitate chromatin remodeling through nucleosome sliding, nucleosome disruption, and exchange of histone components. Although the roles of many ATP-dependent remodeling complexes were first identified in transcriptional regulation, it has recently been shown that some of these complexes (e.g., INO80, RSC, SWI/SNF, and SWR-C) are also recruited for DNA repair.

5. CELL CYCLE CHECKPOINT REGULATION

One of the primary responses to DNA damage besides stimulation of DNA repair is the activation of cell cycle checkpoints. Cell cycle checkpoints are regulatory pathways that control the order and timing of cell cycle transitions and ensure that critical processes at each phase of the cell cycle, such as DNA replication and chromosome segregation, are completed with high fidelity before progressing to the next phase. A timely cell cycle progression results in the correct transmission of genetic information from parent to daughter cells. When stimulated with suitable growth factors, quiescent cells leave the cell cycle's resting phase, called gap 0 (G0), and enter gap 1 (G1) phase, then segue to DNA replication or synthesis (S) phase, which is followed by a second gap (G2) phase, and finally on to cell division or mitosis (M). DNA damage effect on cell cycle is mainly seen in three checkpoints: (1) G1/S (G1), (2) intra-S phase, and (3) G2/M. When DNA damage is sensed, cells arrest the cell cycle at these specific phases by activating the appropriate DNA damage checkpoint(s). For instance, on perturbation of DNA replication by normal, stalled replication forks or by drugs that interfere with DNA synthesis, cells activate the checkpoint that arrests the cell cycle at G2/M transition until DNA replication is complete. Checkpoint pathways also induce transcription of genes that contribute to the repair and its quality control.

Checkpoint activation is mediated by transcriptional and post-transcriptional modifications of proteins that regulate the cell cycle. Such regulation is carried out by oscillations in cycling-dependent kinases (Cdks), which are positively regulated by cyclins (cdk-cyclin complexes) and by dephosphorylation (mediated by the dual specificity Cdc25 phosphatase family, including Cdc25A, Cdc25B, and Cdc25C); Cdks are negatively regulated by Cdk inhibitors and Wee1 and Mik1 kinase-dependent phosphorylation within the ATP-binding domain. The cdk-cyclin complexes influencing G1 progression and the G1/S checkpoint are primarily Cdk4-cyclin D, Cdk6-cyclin D, and Cdk2-cyclin E, whereas Cdk2-cyclin E complexes normally promote the G1/S transition.

Progression into S phase, and transition from G2 into M, is regulated by Cdk2/cyclin A and Cdk1/cyclin B, respectively. During the DNA damage response, activation of ATM/ATR and Chk1 and Chk2 kinases leads to phosphorylation of all three Cdc25 phosphatases. Chk1 or Chk2 phosphorylation of Cdc25 leads to inhibitory sequestration of Cdc25C by 14-3-3 proteins and ubiquitin-mediated proteolysis of Cdc25A. Cdc25A acts earlier in the cell cycle than Cdc25B and Cdc25C, is thought to be important for maximal Cdk

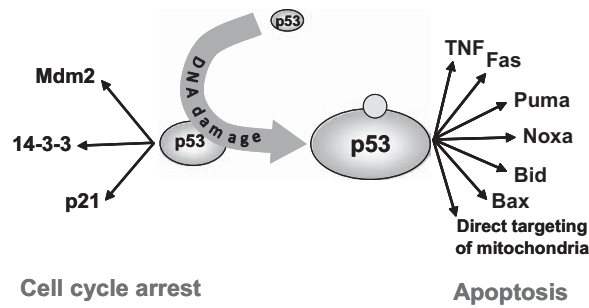


Figure 8-3. p53 is a major player influencing cell fate decisions after DNA damage. On DNA damage, p53 is stabilized and transactivates genes involved in temporary cell cycle arrest. If DNA damage persists, sustained p53 activation and/or particular post-transcriptional modifications (circle) shift p53 function toward activation of proapoptotic factors.

activity during progression through S phase, and also contributes to passage through mitosis. This leads to sustained phosphorylation and inhibition of cdk-cyclin complexes, halting cell cycle progression. Simultaneously, DNA damage activates cdk-cyclin inhibitors (p21, p27, and p57 for cdk2-cyclin E; p16 for cdk4-cyclin D or cdk6-cyclin D), leading to cell cycle arrest.

Multiple kinases activated by DNA damage (i.e., Chk2) were reported to phosphorylate and activate the tumor suppressor protein p53. In turn, p53 transcriptional activity induces proteins that contribute to both cell cycle checkpoint and DNA repair. Among those, notable is p21 transactivation (Figure 8-3), which blocks Cdc2 phosphorylation and inhibits CDK2 and CDK4, leading to G2/M and G1 arrest, respectively. p53 also transactivates 14-3-3, which sequesters Cdc25C in the cytoplasm and promotes the activation of Wee1, a tyrosine kinase that negatively regulates Cdc2, thus blocking entry into mitosis.

Additional G2/M checkpoint regulators include the mitotic serine/threonine Polo-like (Plk) and Aurora-like kinases. Plk1 promotes mitotic entry by phosphorylating and activating Cdc25C and by targeting Wee1 for degradation. In response to DNA damage, Plk1 is inhibited in an ATM/ATR-dependent manner. Inhibition of Plk1 stabilizes Wee1 and maintains Cdks in their inhibited form. Unlike Plk1, Plk3 is involved in G2/M arrest by phosphorylating and inhibiting Cdc25C.

Aurora A kinase is normally required to recruit and activate Cdk1 (cdc2)/cyclin B and to commit cells into M phase. In response to DNA damage, Aurora A kinase inhibition is associated with G2/M checkpoint activation, whereas its over-expression results in checkpoint bypass. Although early studies suggested clear, functional distinctions between DNA repair and damage checkpoint proteins, it now seems certain that many DNA damage response proteins are involved in both processes.

6. WHEN REPAIR FAILS: SENESCENCE VERSUS APOPTOSIS

Activating DNA damage checkpoints enforces growth arrest of damaged cells and allows repair mechanisms to mend the injury. Once repair is complete, cells exit the checkpoints and resume cell cycle progression and functions. However, when damage is severe or irreparable, mitotic cells from renewable tissues rely on one of two mechanisms to avoid replication. They permanently arrest the cell cycle (cellular senescence) or trigger cell death (apoptosis). These two fates are triggered by signal transduction pathways that converge on the p53 tumor suppressor (Figure 8-3). p53 activates downstream effector genes, such as those encoding the cyclin/cdk inhibitor p21 for cell cycle arrest or Puma and Bax for apoptosis. Cell fate after DNA damage depends on a balance between expression of cell cycle arrest or proapoptotic genes. The differential expression of these set of genes is regulated by two distinct, albeit not mutually exclusive, mechanisms. The first is post-transcriptional modification of p53 itself, and the second is regulation of other proteins that constrain the function of p53, such as Mdm2 or Rb. Modifications in p53 likely involve changes in p53 DNA-binding specificity or recruitment of coactivators specific for different classes of these genes. In this respect, some post-translational modifications of p53, such as phosphorylation of S46 or K120 acetylation of p53 by the histone acetyltransferases Tip60 and hMOF allows the recruitment of a coactivator required for transcription of proapoptotic genes. On the other hand, monoubiquitination or acetylation on K320 selectively favors p53 transcriptional activity on cell cycle arrest genes.

6.1. DNA damage response and the induction of apoptosis

As mentioned above, the DDR leads to phosphorylation and stabilization of p53 and subsequent upregulation of p21, which triggers G1/S arrest. For low levels of DSBs, only a minor fraction of p53 is sufficient to drive transcription of the *p21* gene and cause temporary cell cycle arrest. For high levels of DSBs, p53 accumulates and in cooperation with coactivated transcription factors activates proapoptotic factors such as Bax (Bcl-2-associated X protein), PUMA (p53-upregulated modulator of apoptosis), NOXA, Bid, FAS, death receptor 5 (DR5), and PIDD (p53-induced protein with a death domain). It was also shown that the p53 protein itself targets mitochondria, where it associates with and activates the multidomain proapoptotic Bax and Bak proteins. These and others Bcl-2-like proteins exert their apoptotic effect

mainly by increasing mitochondrial membrane permeabilization. Mitochondria serve as a site for convergence of multiple death-inducing stimuli, thereby serving as a pivotal decision center that controls life and death by releasing apoptogenic factors to the cytosol (intrinsic pathway). These death-inducing molecules are located within the mitochondrial intermembrane space and include cytochrome c; DIABLO/Smac, a factor that promotes caspase activation by effecting inhibitors of apoptosis proteins; the nuclease activator apoptosis-inducing factor, Endo G (an apoptotic DNase); HtrA2/Omi (an inhibitor of inhibitors of apoptosis [IAPs] that also contains proapoptotic serine protease activity); and some procaspases. In contrast, Fas and DR5 are members of the tumor necrosis factor receptor family that trigger apoptosis via the extrinsic pathway. PIDD is a caspase-activating protein that binds adaptor protein RAIDD (RIP-associated Ich-1/CED homologous protein with death domain), which in turn binds caspase-2. Thus p53 target genes are capable of activating several apoptosis pathways, although most data argue that the mitochondrial pathway is dominant.

6.2. p53-independent mechanisms of apoptosis

Although most human tumors lose expression of functional p53, they do not lose their ability to undergo apoptosis completely. Cells employ several strategies to trigger p53-independent DNA damage-induced apoptosis. In p53 mutant tumors, the role is also played by other p53 family members such as p63 and p73. p73 is rarely mutated but often over-expressed in tumors. Whereas p53 may require p63 and p73 for triggering apoptosis, p73 is proapoptotic, even in the absence of p53. Apoptosis induced by p73 has been shown to be mediated by transcriptional upregulation of PUMA and NOXA, which in turn provokes Bax mitochondrial translocation and cytochrome c release.

Another protein that influences cellular life/death decisions independent of p53 is the mitogen-activated protein kinase (MAPK) family member c-Jun N-terminal kinase (JNK). JNK controls apoptosis both positively and negatively, depending on cell type, cell context, and stress signal. Proapoptotic JNK substrates comprise both transcription factors (e.g., c-Jun ATF-2, ATF-3, and c-Fos) and proteins that execute apoptosis (e.g., proapoptotic Bcl-2-related proteins). Recently, it was shown that JNK may also contribute to the apoptotic response of cells to activated caspases by phosphorylating H2AX at a non-canonical site that is required for apoptotic DNA fragmentation.

It has been suggested that the strength of survival signals (epidermal growth factor, insulin) determines the pro- or antiapoptotic effect of JNK activation. Alternatively, the role of JNK in cell fate can be regulated by a balance between cell survival signals and proapoptotic stimuli. For example, it was shown that UV-induced apoptosis is repressed by receptor tyrosine kinase-mediated inactivation of Forkhead Box O transcription factor Foxo. The function of JNK might be also governed by a timing mechanism, as shown in *Drosophila*. Whereas short-term activation of JNK (which is normally inhibited by a negative feedback loop involving Puc) would allow cell repair, long-term activation would lead to cell death. Such a time-dependent cellular response to JNK activation has been also observed in mammalian cells.

Whereas it is clear that JNK activation after genotoxic stress occurs through DNA damage-dependent and -independent mechanisms, the relative contribution of each of these mechanisms is still unclear. The main reason for that is that most genotoxins induce both pathways. JNK activation independent of DNA occurs when genotoxic agents activate growth factor receptors or increase reactive oxygen species levels (i.e., UV light). JNK-mediated activation by reactive oxygen species is mediated by the MAPKKK ASK1 (a specific reactive oxygen species target) and Src kinase. JNK activation by UV is also mediated by inhibition of MAPK phosphatase-1 expression.

Although JNK activation by DNA damage is well established, the mechanisms underlying the link between the DNA damage and actual activation of JNK are not as clear. Several studies have shown that JNK activation after DNA damage is mediated by ATM, DNA-PKs, and Cockayne syndrome B. Alternatively, it was proposed that DNA damage per se is sufficient to elicit a signal from the nuclei to the cytosol for JNK activation. The nonreceptor tyrosine kinase c-Abl, for example, is activated after IR and is required for activation of JNK in response to cellular stress. Ras-association domain family 1C (RASSF1C) also participates in the activation of SAPK/JNK after its release to the cytoplasm from the nuclear complex containing Daxx, which is degraded by DNA damage. In all cases the signals will result in the activation of JNK or its upstream kinases MKK4/7 and MEKK1-4.

Interestingly, activating JNK, c-Jun, and ATF2 after genotoxic stress also controls the cellular response to damage by regulating the expression of various DNA repair genes, including *ERCC3*, *XPA*, *RAD23B*, and *MSH2*. Independent of its function as a transcription factor,

ATF2 has been revealed as an important component of the damage response. ATM phosphorylation of ATF2 has been shown to cause its localization into DNA repair foci, where it colocalizes with components of the DNA repair machinery, Rad50, NBS1, and Mre11.

Another pathway induced by DNA damage is the PI3K/Akt pathway. Activation of Akt has clearly an anti-apoptotic effect that can be considered as a compensatory protective mechanism activated by the cell to escape death. Several components of the PI3K/Akt pathway have been reported to be phosphorylated in the DNA damage response, particularly Akt at Ser473. This phosphorylation, critical for Akt activity, was shown to be mediated by DNA-PK and ATM. Activated Akt then participates in an elaborate control system deciding between cell survival and death by counteracting the role of p53. Akt mediates phosphorylation and nuclear translocation of Mdm2 and inhibits interaction between Mdm2 and p19^{ARF}, thereby potentiating the activity of Mdm2 in degrading p53. Akt can phosphorylate Chk1 and reduce the nuclear localization of Chk1, thereby interfering with Chk1-mediated p53 phosphorylation and subsequent p53 stabilization. In turn, Akt activity is controlled by p53 via transactivation of phosphatase and tensin homolog deleted in chromosome ten (PTEN) and the Akt inhibitor 14-3-3 and by repressing the catalytic subunit of PI3K.

Several evidences indicate that the transcription factor nuclear factor kappa B (NF- κ B) plays a critical role in the cellular response against a variety of DNA-damaging agents. Most genotoxins transiently activate NF- κ B by inducing pathways similar to those activated by cytokines (e.g., mediated by either I κ B kinase complex activation or IKK/NF- κ B-inducing kinase activation). On the other hand, IR and some chemotherapeutic agents induce an atypical pathway that results in a slower and more stable (approximately 4 hours) NF- κ B activation that is dependent on ATM. IR activates NF- κ B by increasing association of NF- κ B essential modulator (NEMO) with IKK α and IKK β , an essential step for I κ B phosphorylation and subsequent degradation. The effects of DSB on NEMO are mediated by increasing NEMO sumoylation, mono-ubiquitination, and ATM-dependent phosphorylation. After I κ B degradation, NF- κ B (mostly p50/p65 dimer) moves to the nucleus and, depending on the nature of the genotoxic signal and the cell type, regulates transcription of either pro- or anti-apoptotic genes. UV activates another atypical pathway of activation of NF- κ B. This pathway is independent of IKK and would involve Ck2-mediated phosphorylation of I κ B.

In summary, DNA damage-triggered signaling and execution of apoptosis are cell-type and genotoxin-specific events that depend on p53 (p63 and p73) status, death-receptor responsiveness, MAPK activation (as well as Akt and NF- κ B activation) and, most importantly, DNA repair capacity.

6.3. DNA damage response and senescence induction

Premature senescence is induced by agents that damage DNA or disrupt heterochromatin or by over-expressed oncogenes. Engagement of the DNA damage response by genotoxins was described previously in this chapter. Oncogene expression leads to elevated intracellular levels of reactive species, augmented numbers of active replicons, alterations in DNA replication fork progression, and the appearance of DNA single- and double-strand breaks that initiate damage responses.

Senescent cells are typically characterized by a large, flat morphology, expression of a senescence-associated β -galactosidase (SA β -gal) activity of unknown function, and senescence-associated heterochromatin foci. The foci contain modifications and associated proteins characteristic of transcriptionally silent heterochromatin, such as methylated lysine 9 of histone H3 (H3K9Me), heterochromatin protein 1 (HP1), the histone H2A variant macroH2A, and the high-mobility group A (HMGA) proteins.

Mechanistically, DNA damage-induced growth arrest depends on the functional status of Rb via the p53–p21–Rb pathway or via p53-independent Rb pathways, either p16-dependent or -independent. In senescent cells, p21 and p16 play a critical role by inhibiting CDK-dependent Rb phosphorylation, leading to transcriptional repression of E2F target genes necessary for DNA synthesis and cell cycle progression. The subsequent orchestration and temporal requirements of senescence-associated markers and cell cycle regulators such as p16^{INK4a}, p21^{Cip1}, CDK4, ARE, p53, or PML to induce and maintain senescence are not fully understood and seem, at least to some extent, to be context, cell type, and species dependent.

Senescence, induced by the damage response present in early premalignant tumors (likely triggered by oncogene-induced DNA damage), plays a protective function by raising a barrier to tumor progression. However, it has been speculated that senescence may produce a selective pressure that eventually favors outgrowth of malignant clones containing genetic or epigenetic defects in the genome maintenance machinery.

7. DNA DAMAGE FROM OXIDATIVE STRESS

Reactive oxygen species, generated as by-products of cellular metabolism (i.e., energy produced from mitochondria), are part of an antimicrobial or antiviral response, as are detoxification reactions carried out by the cytochrome P-450 system. Both antitumor and environmental agents (e.g., UV, IR, redox chemicals, and cigarette smoke) also readily generate reactive oxygen species. Oxidative stress occurs when levels of reactive oxygen species exceed the body's natural antioxidant defense mechanisms.

The result is damage to biomolecules such as lipids, proteins, and DNA. Reactive oxygen species include superoxide anion radical (O_2^-), singlet oxygen (O_2), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical (OH^-). Superoxide and hydrogen peroxide are normally not reactive toward DNA. However, in the presence of ferrous or cuprous ion, both superoxide and hydrogen peroxide are converted to highly reactive hydroxyl radicals that induce multiple DNA modifications. DNA damage induced by reactive oxygen species includes a range of specifically oxidized purines and pyrimidines, alkali labile sites, single-strand breaks, base and nucleotide modifications, and instability formed directly or by repair processes. Reactive oxygen species also induce a number of covalent modifications to DNA, such as inter- and intrastrand cross-links and protein-DNA cross-links.

Because some of these lesions possess mutagenic properties, they may lead to carcinogenesis if not repaired. An oxidized form of guanine, 8-hydroxydeoxyguanosine, is readily bypassed by DNA polymerase and is the major oxidative product resulting from damaged DNA that produces mutations (A:T to C:C or G:C to T:A transversion mutations) because it pairs with either adenine or cytosine bases. In human tumors, G to T transversions are the most frequent mutations found on the p53 suppressor gene. Oxidative modification of DNA is repaired by a ubiquitous base-excision repair pathway. At least in mammals, the response to oxidative stress involves p53. Recent studies reveal that reactive oxygen species act as both an upstream signal that activates p53 and as a downstream factor that mediates apoptosis and growth arrest. Thus DNA damage induced by reactive oxygen species leads to p53 activation, growth arrest, and apoptosis.

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9

Ceramide and Lipid Mediators in Apoptosis

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1. INTRODUCTION

As a cellular signaling program, apoptosis is a highly controlled and complex process that depends on the orchestrated interactions of multiple soluble factors: ions (e.g., Ca^{2+}), proteins (e.g., caspases, Bcl-2 family members), and nonprotein substrates (e.g., DNA). Equally important, although less well characterized, is signaling through cellular membranes and the lipids and proteins contained therein. Lipids are the primary constituents of biological membranes and thus play a structural role in defining cellular and organellar boundaries. However, lipids are not merely passive molecules serving inert, structural functions in these membranes. Many lipids are now appreciated as signaling molecules, capable of influencing diverse cellular processes and exerting powerful influence over many physiologic and pathophysiologic processes, such as programmed cell death. Sphingolipids represent one class of bioactive lipid mediators that are now recognized as key determinants of cell fate. This chapter discusses the regulated generation of bioactive sphingolipids (e.g., ceramide) and how sphingolipid signaling impacts the regulation of programmed cell death.

Lipid signaling is the control of cellular function through the modulation of membrane lipid composition. Although a full discussion of cellular lipid composition would require its own textbook, a few general concepts should be presented. In most metazoan cells, the predominant classes of lipids are the glycerolipids, sphingolipids, sterols, and eicosanoids. Major glycerolipid species include phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine (PS), and phosphatidylinositol, and important minor species are diacylglycerol (DAG), phosphatidic acid, lysophosphatidic acid, and the phosphatidylinositol phosphates

(PIPs). Sphingomyelin (SM) and glycosphingolipids (GSL), such as glucosylceramide (GlcCer) and gangliosides, make up the bulk of the cellular sphingolipid repertoire. Ceramide is comparatively less abundant, and sphingosine and sphingosine-1-phosphate (S1P) are found at even lower levels. Of the sterols, cholesterol is the most abundant. Finally, the highly diverse class of lipids known as eicosanoids (i.e., metabolites of arachidonic acid) are involved in the regulation of a multitude of physiologic processes – most notably, inflammation. Clearly, for cellular signaling, the eukaryotic cell has an immense array of lipids at its disposal.

Cells accomplish signaling through lipids via several mechanisms, but the simplest signaling paradigm is based on the production of a bioactive lipid from an inert, high-abundance precursor. For example, in G protein-coupled receptor signaling, phosphatidylinositol-(4,5)-phosphate (PIP_2) is hydrolyzed by phospholipase C (PLC) to form DAG and inositol-(3,4,5)-triphosphate (IP_3). DAG proceeds to bind to protein kinase C (PKC), recruiting it to the membrane, allowing its activation, and promoting a signaling cascade. Other bioactive lipid/precursor pairs are included in [Table 9-1](#). However, it must be understood that the terms *bioactive* and *inert* are relativistic and depend on the context and biology in question.

Many lipids are involved in the regulation of cell death. Lipids such as DAG, phosphoinositides, and S1P generally oppose proapoptotic pathways, whereas lipids such as ceramide and sphingosine can promote these pathways. Although tremendously important in the regulation of cell fate, lipid-mediated pathways of cell growth and survival (e.g., phosphoinositide-3-kinase [PI3K]/Akt pathways, sphingosine-1-phosphate receptor signaling) are not discussed at length in this chapter. Another topic that is not elaborated on is that of PS

Table 9-1. Signaling lipids and their precursors

Precursor of signaling molecule(s) (greater abundance)	Signaling molecule(s) (lesser abundance)
PIP ₂	DAG, IP ₃
SM	Ceramide
Glucosylceramide	Ceramide
Phosphatidic acid	Lysophosphatidic acid
Ceramide	Ceramide-1-phosphate Sphingosine
Sphingosine	Sphingosine-1-phosphate
Arachidonic acid-containing glycerophospholipids	Eicosanoids

exposure on the outer leaflet of the plasma membrane and its role in the recognition of apoptotic cell fragments by macrophages. Instead we focus on the lipids and pathways that have been shown to play largely proapoptotic signaling roles.

In this chapter, we mostly examine the roles of ceramide in the regulation of apoptosis. The aims of this

chapter are to (1) introduce the pertinent sphingolipids, metabolic enzymes, and basic properties and precepts essential for understanding the complex role of sphingolipids as signaling molecules; (2) review the evidence supporting a role for sphingolipids in the apoptotic program; (3) highlight studies that illustrate the vital role of ceramide in apoptosis-related disease; and (4) present a few of the many remaining unanswered questions concerning sphingolipids in cell death.

2. SPHINGOLIPID METABOLISM: CONSTITUENTS, COMPARTMENTALIZATION, AND KEY CONCEPTS

The synthesis of all sphingolipids depends on the de novo formation of ceramide, which occurs by a series of catalytic steps (Figure 9-1). The rate-limiting step of sphingolipid synthesis occurs when serine and palmitoyl-CoA are condensed by the enzyme serine palmitoyltransferase (SPT) to form 3-ketosphinganine – a transient metabolite that is readily converted to dihydrosphingosine (also known as sphinganine). Dihydrosphingosine is then subject to acylation by ceramide synthases (CerSes). Fatty acyl chains are transferred from

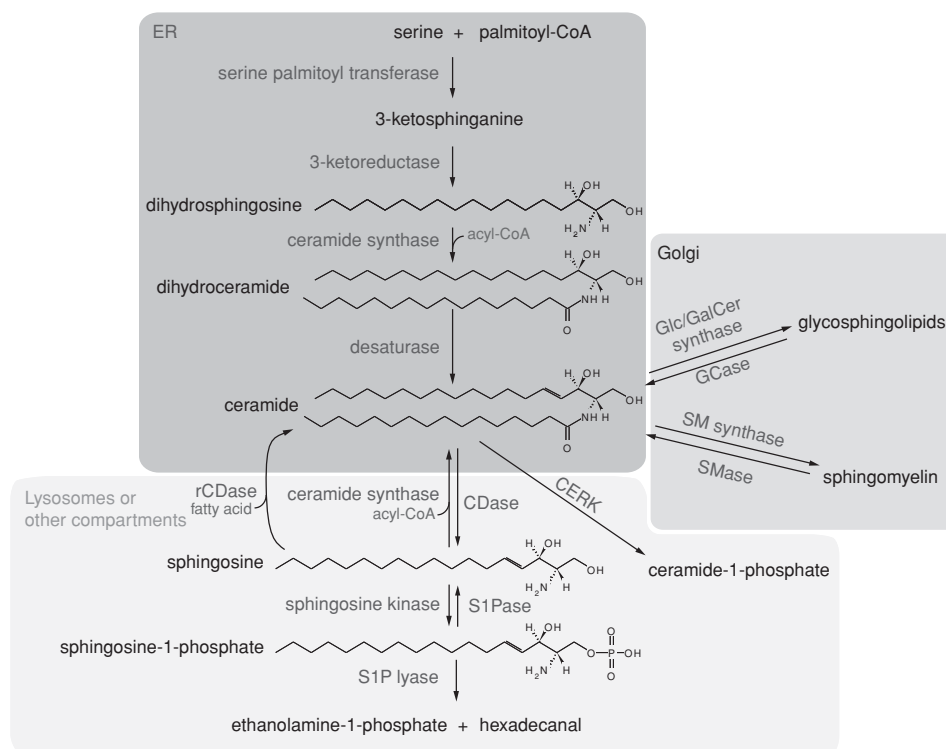


Figure 9-1. Sphingolipid metabolism. Serine and palmitoyl-CoA are condensed by SPT to form 3-ketosphinganine, which is subsequently metabolized to dihydrosphingosine. Dihydrosphingosine is a substrate for acylation by CerS, producing dihydroceramide. Dihydroceramide desaturase reduces dihydroceramide to form ceramide. Ceramide is then trafficked to the Golgi apparatus, where it is the substrate for the synthesis of more complex sphingolipids. Although not exclusively, the breakdown of complex sphingolipids can proceed via lysosomal pathways, which ultimately result in the production of free sphingosine. Sphingosine can be the substrate for either CerSes or sphingosine kinases to form ceramide and S1P, respectively.

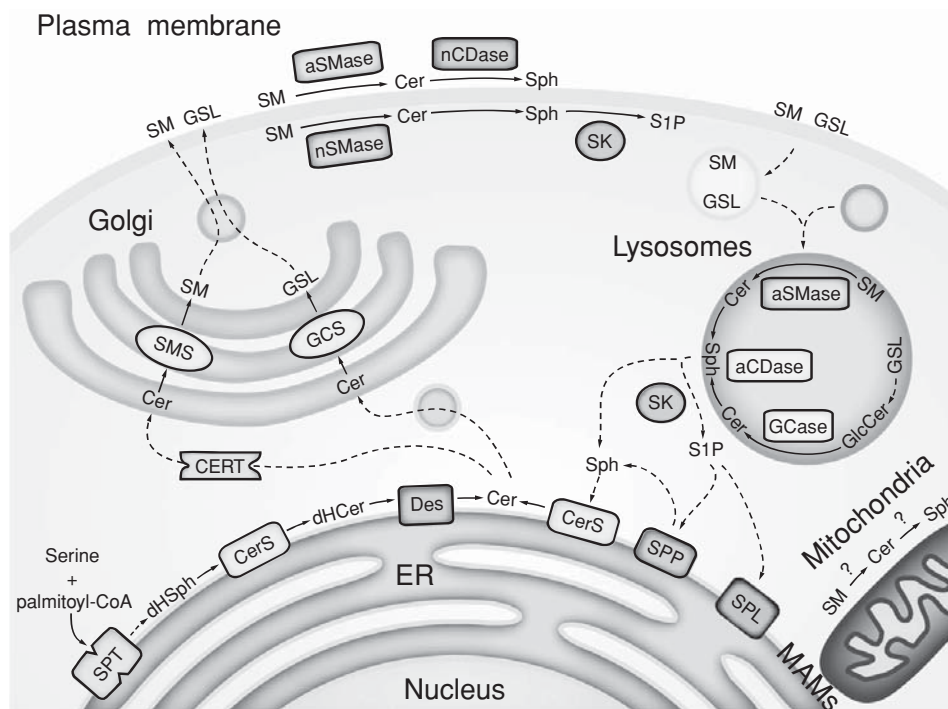


Figure 9-2. Compartmentalization of sphingolipid metabolism. Most enzymes of de novo sphingolipid synthesis reside in the ER. Here, ceramide is formed and then transported to the Golgi apparatus by vesicular trafficking, as well as nonvesicular transport via the ceramide transfer protein CERT. In the Golgi, ceramide is metabolized to complex sphingolipids, which are distributed to the plasma membrane and the endosomal compartments. Degradation and recycling of sphingolipids proceeds through endocytosis and transfer to the lysosomes, where many sphingolipid-metabolizing enzymes reside. Lysosome-derived sphingosine may be recycled into ceramide directly, or it may be converted by SK to the more hydrophilic S1P and subsequently dephosphorylated by S1P phosphatase. S1P may also be degraded via S1P lyase at the ER. aCDase, acid ceramidase; aSMase, acid sphingomyelinase; Cer, ceramide; CERT, ceramide transfer protein; dHCer, dihydroceramide; dHSph, dihydrosphingosine; ER, endoplasmic reticulum; GCS, glucosylceramide synthase; GSL, glycosphingolipids; nSMase, neutral sphingomyelinase; MAMs, mitochondria-associated membranes; S1P, sphingosine-1-phosphate; SM, sphingomyelin; SK, sphingosine kinase; SMase, sphingomyelinase; SMS, SM synthase; Sph, sphingosine; SPP, S1P phosphatase; SPT, serine palmitoyltransferase. See Color Plate 8.

acyl-coenzyme (acyl-CoA) onto the free amine group of dihydrosphingosine producing dihydroceramide. Dihydroceramide is subsequently reduced by dihydroceramide desaturase to form ceramide.

The cellular compartmentalization of sphingolipid metabolism is as important as the individual biochemical reactions (Figure 9-2). The early steps of sphingolipid biosynthesis are largely confined to the endoplasmic reticulum (ER). With some exceptions, most enzymes (e.g., SPT, CerSes) of the de novo synthetic pathway are found exclusively in this organelle. Ceramide formed in the ER must then be transferred to the Golgi complex via vesicular and nonvesicular trafficking for metabolism into complex sphingolipids such as SM, glucosylceramide, and gangliosides. The degradation of complex sphingolipids proceeds primarily in the endo-lysosomal compartment, yielding free sphingosine. Sphingosine can be phosphorylated to S1P, or it may be re-acylated to form ceramide – a process known as the salvage pathway.

Although a detailed assessment of the many complexities of sphingolipid metabolism is beyond the scope of this chapter, several key concepts should be emphasized. First, and most apparent from the metabolic scheme, is that ceramide occupies a central position in sphingolipid metabolism and represents a “hub” in sphingolipid synthesis, degradation, and interconversion. As a metabolic intermediate, ceramide may seem like an unlikely candidate for a signaling molecule. However, when one considers the compartmentalization of the enzymes of ceramide metabolism, it becomes apparent that ceramide is uniquely positioned to behave as a signaling lipid. Because ceramide is both substrate and product of multiple enzymes, its levels represent a balance between synthetic and degradative processes that must be highly regulated. Furthermore, multiple sphingolipid enzymes, including sphingomyelinases (SMases), CerSes, and ceramidases (CDases), exhibit unique localization and allow for ceramide levels to

Box 9-1. Methods of sphingolipid analysis I: Diacylglycerol kinase assay

The diacylglycerol kinase (DGK) assay has been long used as a means of measuring ceramide and continues to be the standard in many labs today. In this assay, ceramides are extracted and labeled with [γ - ^{32} P]ATP at the 1-OH position using the diacylglycerol kinase from *Escherichia coli*. Labeled ceramides are analyzed by thin-layer chromatography (TLC), and radioactivity can be measured through scintillation counting. Although relatively straightforward, drawbacks of the standard DGK assay include the requirement for radioisotopes, the inability to easily distinguish dihydroceramide from ceramide, and the lack of resolution of specific ceramide species (e.g., C₁₆- vs. C₁₈-ceramide). Sphingolipids are also measured by metabolic labeling using radiolabeled sphingolipid precursors. For example, cells can be incubated with either [^{3} H]-serine or [^{3} H]-palmitate to label sphingolipids and determine metabolic flux through the de novo pathway. Radiolabeled dihydrosphingosine or sphingosine can be used to track metabolic flux through CerS or SK. Like the DGK assay, experiments using labeling require extraction of lipids and analyzed by TLC and scintillation counting.

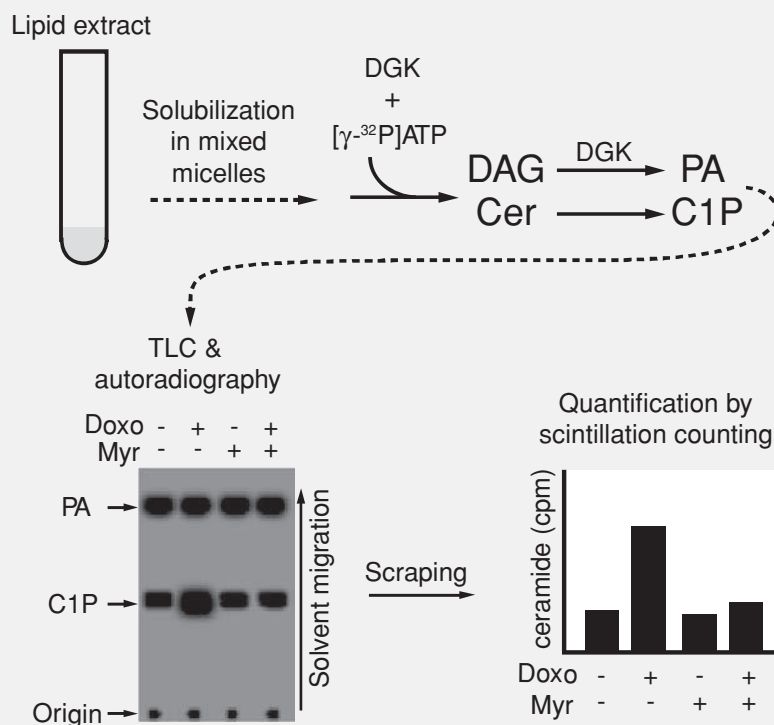


Figure B9-1. Schematic representation of DGK method for ceramide quantification. Lipid extracts are prepared from any source, and samples and standards are reconstituted in detergent-containing micelles and incubated with DGK and [γ - ^{32} P]ATP. The lipids are separated by TLC. Bands corresponding to ceramide-1-phosphate (C1P) are scraped, and radioactivity is quantified by scintillation counting.

be differentially regulated in the subcompartments of the cell. Thus the concept of compartmentalization of ceramide metabolism emerges as a second and key concept in analyzing ceramide function. Accordingly, ceramide function needs to be considered in a pathway-specific and compartment-specific manner, which is further corroborated by the molecular heterogeneity of ceramide species that may localize to distinct compartments.

The third key concept – that of metabolic flux – also relies on an understanding of the various sphingolipid enzymes and their compartmentalization. On detecting elevations in ceramide, it is insufficient to consider just one enzyme as a source of ceramide; instead, the cause(s) of ceramide accumulation must be addressed both in terms of its generation (e.g., from

SM hydrolysis) and its catabolism (e.g., by a ceramidase). Although such consideration has been previously arduous from an experimental perspective, new tools and knowledge of the multiple sphingolipid enzymes are making complex sphingolipid analyses possible (Boxes 9-1 and 9-2).

3. SPHINGOLIPIDS AS MEDIATORS OF APOPTOTIC SIGNALING

The current synthesis of the role of sphingolipids in the regulation and execution of cell death signaling incorporates 17 years of research and more than 2,500 publications. An understanding of the role of ceramide in cellular signaling first requires an understanding of signaling paradigms. Basic cellular signaling involves the

Box 9-2. Methods of sphingolipid analysis II: High-performance liquid chromatography and mass spectrometry

The introduction of high-performance liquid chromatography (HPLC) coupled with mass spectrometry (LC/MS) to the measurement of sphingolipid levels has allowed investigators to study ceramide and other sphingolipids in greater detail. In this method, lipid samples and standards are separated using reverse phase chromatography, and fractions are analyzed by mass spectrometry. Individual sphingolipids are identified by both retention time in the column as well as their mass transitions, and, using calibration standards, the sphingolipids may be quantified. The advantage of LC/MS lies in the ability to simultaneously quantify multiple sphingolipid species (e.g., ceramide, sphingosine, and S1P) as well as ceramide subspecies with particular acyl chain lengths (e.g., C_{16} -ceramide vs. C_{18} -ceramide).

Using this technology, it has been found that certain subspecies of ceramide (e.g., C_{16} -ceramide, C_{18} -ceramide, $C_{24,1}$ -ceramide) may differentially regulate cell death. After induction of apoptosis, ceramide species accumulate at different rates or to differing degrees, and some studies suggest that certain species, but not others, are associated with the promotion of apoptosis. In head and neck cancer cell lines, for example, the production of C_{18} -ceramide by CerS1 was linked to caspase activation and cell death, whereas C_{16} -ceramide, a product of CerS5 and CerS6, failed to exhibit the same association. Although the significance of these findings remains a matter of speculation, the data suggest that there may be specific roles for individual ceramide species in regulating cell death.

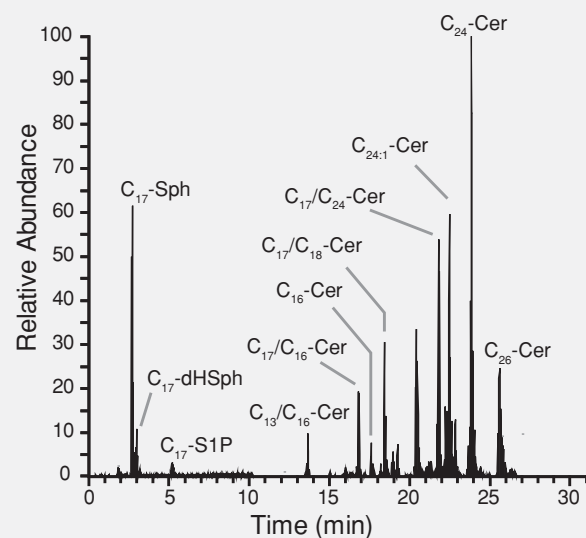


Figure B9-2. LC/MS detection of ceramide species. Example chromatogram of ceramides and sphingoid bases extracted from MCF-7 adenocarcinoma cells. Unnatural, synthetic sphingolipid species containing C_{13} - or C_{17} -sphingoid bases (e.g., C_{17}/C_{16} -ceramide) are used as internal standards for HPLC separation. Most mammalian ceramides contain C_{18} -sphingoid bases and are therefore distinguishable from the synthetic standards by their retention time as well as mass transition. Using ceramide standards to generate calibration curves, ceramides are quantified and normalized to total protein or total lipid phosphate.

reception of a stimulus, the production and activation of signaling intermediates, the activation of effectors, and a resulting change in cell behavior (Figure 9-3). In the case of programmed cell death, there is a multitude of potential stimuli, and although the “reception” phase of cell death signaling may be divergent in response to various stimuli, at some level many signaling pathways converge to produce a seemingly unified response – apoptosis. A number of proapoptotic signaling modalities are engaged to bring about apoptosis, including Ca^{2+} release, production of lipid mediators, and the activation of proteases.

3.1. Basic cell signaling often involves small molecules

Most cellular signaling uses small molecules to convey information between protein components of each pathway. For example, Ca^{2+} signaling can occur by

releasing Ca^{2+} from compartments such as the ER or extracellular milieu where Ca^{2+} concentrations are relatively high. After stimulation and activation of Ca^{2+} channels, elevations in cytosolic Ca^{2+} can cause the modulation of multiple downstream effectors. In a similar fashion, signaling sphingolipids such as ceramide can be released from “storage” in the form of SM at the plasma membrane to produce local changes in the concentration of ceramide. However, unlike soluble free Ca^{2+} , the hydrophobic nature of ceramide confines it to the membrane, where it can interact with membrane proteins (e.g., receptor signaling complexes) or soluble proteins that might be recruited from the cytosol (e.g., protein phosphatases).

3.2. Sphingolipids are cell-signaling molecules

The study of sphingolipids as signaling molecules originally emerged from the finding that sphingosine inhibits

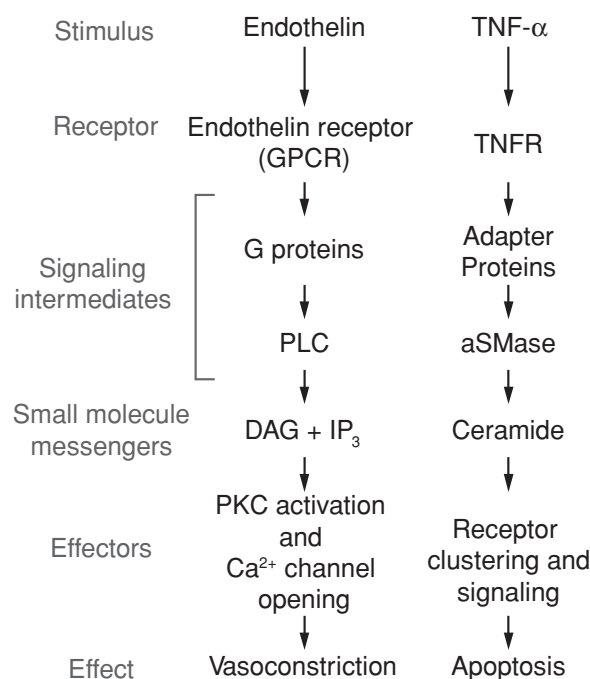


Figure 9-3. A simple signaling paradigm. The most conceptually simple lipid signaling paradigms involve ligand-induced activation of a receptor complex, the recruitment of signaling proteins, and the activation of enzymes that produce signaling lipids. These lipids either recruit proteins to the membrane from the hydrophilic compartments or bind to proteins within the membranes themselves. Lipid-protein interactions result in changes in effector protein function, leading to the downstream activation of signaling components and a resultant biological response. TNFR, tumor necrosis factor receptor; GPCR, G-protein coupled receptor.

the activation of PKC both *in vitro* and as a component of signaling induced by the pleiotropic proinflammatory cytokine tumor necrosis factor- α (TNF- α). Additional studies found that TNF- α could induce ceramide production via SM hydrolysis and that SM-derived ceramide could also modify cell behavior. Thus the field of sphingolipid signaling was born. Once the connections between TNF- α signaling and ceramide were established, it was not long before this lipid was being studied in the context of apoptosis. Although many questions remain today, three main lines of evidence support the hypothesis that ceramide is a key mediator of programmed cell death.

3.2.1. Ceramide induces apoptosis

One of the first connections made between ceramide and apoptosis was the discovery that short-chain, cell-permeable analogs of ceramide (e.g., *N*-acetyl-sphingosine, or C_2 -ceramide) were able to induce apoptosis in leukemia cell lines. The closely related molecule C_2 -dihydroceramide, however, was unable to induce apoptosis, suggesting that the effect was highly specific to the molecular configuration of ceramide.

Several studies followed to establish the importance of ceramide's 4–5 *trans* double bond for its proapoptotic effects. Short-chain ceramides (C_{2-8}) induce apoptosis that involves activation of members of the mitogen-activated protein kinase (MAPK) family (e.g., p38-MAPK and c-Jun *N*-terminal kinase), although the dependence on these signaling pathways is controversial. Short-chain analogs also modulate the activities of protein phosphatases such as PP1 and PP2A that can function to de-phosphorylate prosurvival proteins such as Akt. In addition to its direct effects, C_6 -ceramide, and to a lesser extent C_2 -ceramide, can be metabolized by the salvage pathway to form long-chain ceramides. Moreover, C_6 -ceramide can serve as a substrate for the ceramide transfer protein CERT and thus may modify levels of endogenous ceramide.

Currently, many researchers use C_2 - and C_6 -ceramide applied exogenously to induce cell death, but several other forms of ceramide can also reproduce similar effects. In some cell types, the addition of the bacterial sphingomyelinase from *Bacillus cereus* is sufficient to produce ceramide and cause cell death. Long-chain ceramides (e.g., C_{16} -ceramide) dissolved in appropriate solvent mixtures such as dodecane/ethanol are also able to promote apoptosis. Interest in the proapoptotic abilities of ceramide have led to the development of ceramide analogs and ceramide-containing liposomes for clinical use in the treatment of a variety of diseases.

3.2.2. Ceramide accumulates during programmed cell death

After stimulation of cells in culture with a death-promoting ligand such as TNF- α or genotoxic stress such as doxorubicin, one or more SMases are activated, leading to the hydrolysis of SM and accumulation of ceramide (Figure 9-4). These events typically happen within the first 30 minutes after stimulation, and the activities of the SMases often return to basal levels within 1 hour (Figure 9-5). However, prolonged stimulation results in a second wave of ceramide production that, although less well characterized, often depends on *de novo* ceramide synthesis (e.g., CerS activation) (Figure 9-6). Both SMase- and *de novo*-mediated ceramide production have been described for a variety of diverse cell-death stimuli (Figure 9-7). Details about these two means of ceramide production, as well as their significance in cell death, are provided later in the chapter.

3.2.3. Inhibition of ceramide production alters cell death signaling

In many experimental models of apoptosis, death-induced ceramide production can be inhibited using

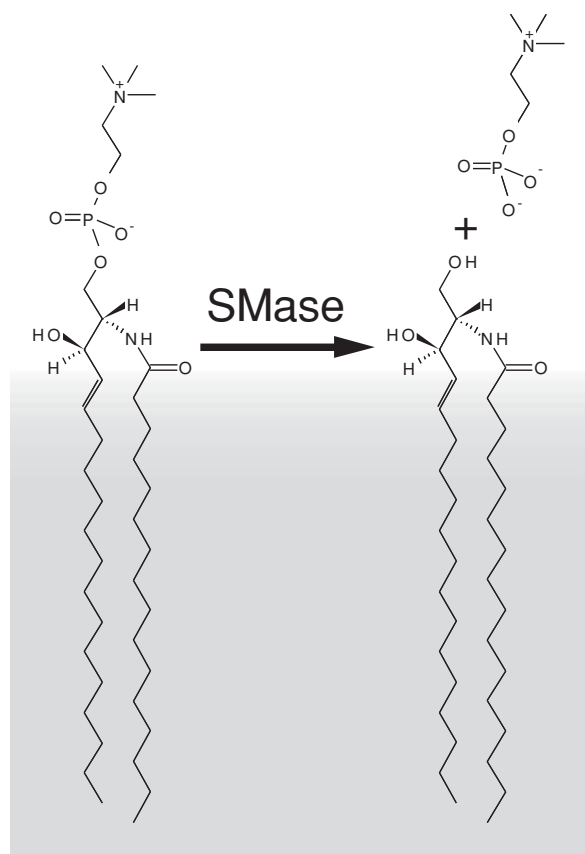


Figure 9-4. Ceramide generation via SMase activation. Activation of SMases leads to hydrolysis of SM to form ceramide and phosphocholine.

pharmacological, genetic, or more recent RNA interference-based approaches. In many cases, such inhibition impedes the cell death process. For example, activation of CD95 on lymphocytes leads to SMase activation, ceramide production, receptor clustering, and apoptosis. Cells deficient in a particular SMase (see [Sections 4.2 and 4.3](#)) fail to achieve the same responses and are protected from cell death. In other systems, inhibition of de novo ceramide synthesis using pharmacological inhibitors of SPT or CerS prevents apoptosis. As we discuss next, cell death signaling can occur via multiple ceramide-mediated pathways that differ in terms of time, subcellular localization, and involvement of particular enzymes of sphingolipid metabolism.

4. CERAMIDE MEDIATES APOPTOTIC CELL DEATH: ROLE OF PARTICULAR ENZYME SYSTEMS

To illustrate the salient features of regulated sphingolipid metabolism and the ceramide-mediated death signaling, we discuss selected studies that highlight key features regarding the nature of sphingolipids as signaling molecules in cell death.

4.1. Ceramide is generated through SM hydrolysis

Hydrolysis of SM by SMases involves cleavage of the phosphodiester bond of SM releasing the phosphorylcholine head group to generate ceramide in a single, rapid step ([Figure 9-4](#)). By virtue of its capacity to generate ceramide acutely, the SMase/ceramide pathway is commonly studied in the context of acute cellular signaling. There are several mammalian SMases that are classified by pH optima for enzymatic activity and requirement for divalent cations. Of the putative mammalian sphingomyelinases that have been identified and cloned, acid sphingomyelinase (aSMase, *SMPD1*) and Mg^{2+} -dependent neutral sphingomyelinase 2 (nSMase2, *SMPD3*) have been implicated in regulated SM hydrolysis in response to a range of stress stimuli. Although elevations in both nSMase2 and aSMase activity have been reported in response to apoptotic mediators, the involvement of nSMase2 in apoptosis has not been well defined. In contrast, there is abundant evidence linking the aSMase/ceramide pathway to apoptotic signaling.

aSMase catalyzes the cleavage of SM to ceramide at an optimum pH of 4.5 to 5.5, befitting its localization within the acidic endo-lysosomal compartment. Deficiency of aSMase results in Niemann-Pick disease (NPD), a lysosomal storage disorder characterized by multiple organ defects and, at the cellular level, by accumulation of SM within lysosomes. In the mid-1990s, as ceramide was gaining attention as a bioactive lipid, it was discovered that cells derived from NPD patients and aSMase knockout mice were resistant to stress-induced apoptosis in response to a variety of stimuli. Cells and

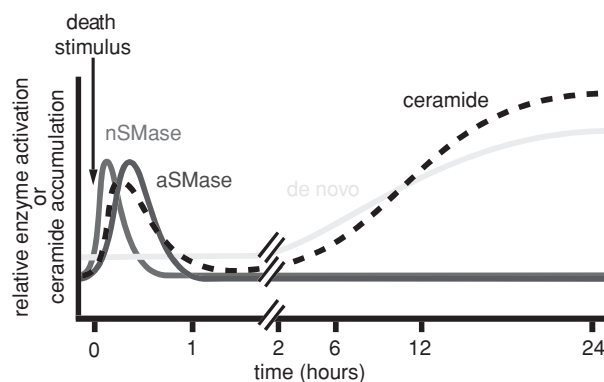


Figure 9-5. Time dependence of ceramide accumulation in cell death. Besides a few exceptions, the time course of enzyme activation and ceramide accumulation appears to differ between each particular enzyme system. Activation of nSMase and/or aSMase occurs within minutes of stimulation, whereas de novo synthesis is increased after several hours.

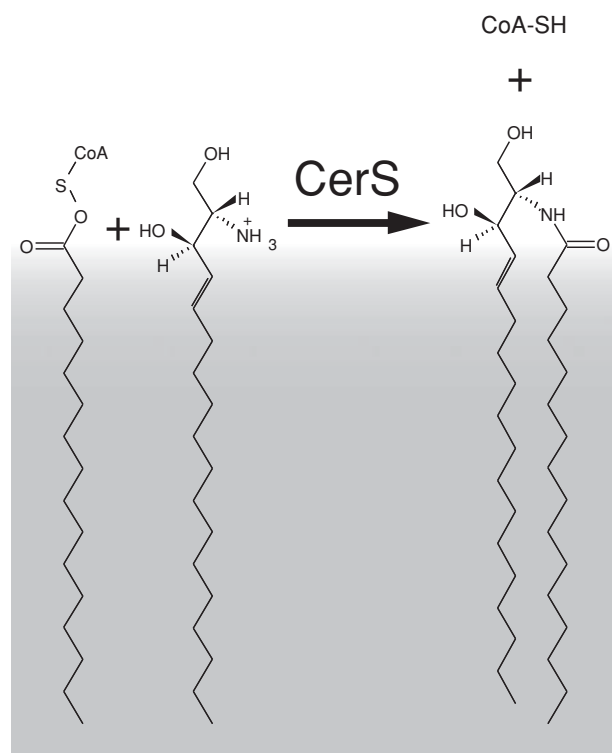


Figure 9-6. Ceramide generation via CerS activation. Ceramide can accumulate when CerS are activated, leading to enhanced ceramide synthesis from dihydrosphingosine (via dihydroceramide, not pictured) or sphingosine.

tissues deficient in aSMase exhibited enhanced survival in response to multiple inducers of cell death, whereas wild-type counterparts underwent apoptosis. Protection from cell death in aSMase-deficient cells in response to receptor-mediated and receptor-independent death signals was attributed to impaired ceramide generation, rather than accumulation of SM, supporting a positive role for aSMase-derived ceramide in the induction of stress-induced apoptosis. Furthermore, many of these same death-inducing stimuli have been shown to induce relocalization of aSMase from the endo-lysosomal compartment to the outer leaflet of the plasma membrane, a rich source of sphingomyelin. This form of aSMase at the plasma membrane is considered crucial for both receptor-mediated and receptor-independent cell death signaling, although the mechanism of relocalization and the precise molecular identity of this “activated” form of aSMase remain poorly defined.

4.2. aSMase is activated after activation of extracellular receptors to promote apoptosis

TNF- α has established roles in the regulation and pathophysiology of inflammatory processes, but high levels of TNF- α can induce cell death in some cell types. One of the seminal studies demonstrating an *in vivo* role for aSMase in TNF- α /TNF receptor-mediated apoptosis defined a role for aSMase in endotoxic shock syndrome. Intraperitoneal administration of the lipopolysaccharide (LPS; also known as endotoxin), a lipid from Gram-negative bacteria, induces disseminated endothelial cell apoptosis. In response to LPS, loss of endothelial integrity causes damage to the lung, intestine, fat, and thymus. Within these tissues, such damage is preceded by ceramide formation. Similarly, direct administration of TNF- α is also capable of inducing ceramide generation within several hours. Blocking TNF- α action using an inhibitory peptide protected animals from LPS-induced ceramide generation and endothelial cell apoptosis, supporting the notion that TNF- α is an intermediary of LPS-induced signaling. Interestingly, aSMase knockout mice are resistant to the effects of LPS despite a normal elevation in TNF- α , suggesting that aSMase is a crucial mediator of the apoptotic response downstream of TNF- α elevation. Lastly, direct injection of TNF- α is capable of inducing endothelial apoptosis in wild-type mice, whereas aSMase-deficient mice are markedly protected, proving that the aSMase/ceramide pathway mediates LPS/TNF- α -induced ceramide generation and subsequent endothelial cell death.

The aSMase/ceramide pathway is also a crucial player in TNF- α -mediated liver disease. Osawa et al. (2005) demonstrated that TNF- α -induced hepatocyte

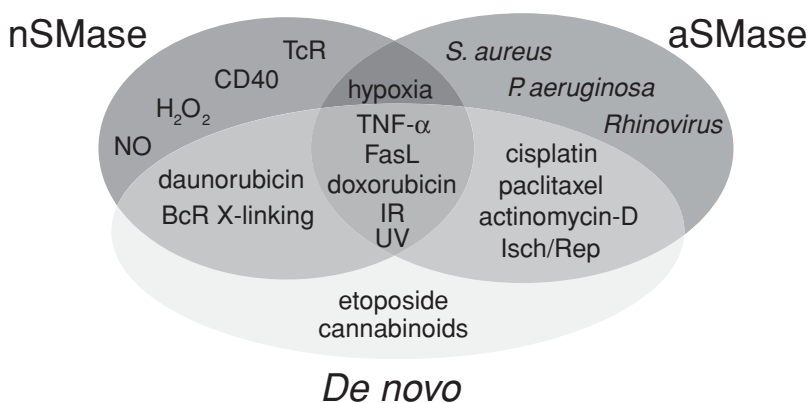


Figure 9-7. Multiple proapoptotic stimuli activate ceramide production and with different kinetics. Depending on the stimulus, ceramide accumulation has been attributed to the activation of several enzyme systems, most notably activation of nSMase, aSMase, or the de novo pathway. NO, nitric oxide; TcR, T-cell receptor; BcR X-linking, B-cell receptor cross-linking; UV, ultraviolet light; Isch/Rep, ischemia/reperfusion injury

death was preceded by ceramide accumulation that was not seen with genetic or pharmacological depletion of aSMase. Additionally, administration of exogenous aSMase restores the cell death in aSMase-deficient hepatocytes in response to TNF- α . Adenoviral-mediated expression in the liver of the cDNA of neutral ceramidase (nCDase), an enzyme that metabolizes ceramide to sphingosine, protected mice from TNF- α -induced elevations in ceramide and liver damage. Over-expression of nCDase also resulted in increased activation of the PI3K/Akt prosurvival pathway, presumably shunting sphingosine through sphingosine kinase and resulting in enhanced levels of the prosurvival sphingolipid S1P. Similar studies have demonstrated a capacity of ceramidase and sphingosine kinase to modulate cell fate, leading some researchers to use the term *rheostat* to describe a hypothetical regulatory mechanism that specifically balances levels of proapoptotic ceramide and antiapoptotic S1P.

Another cell surface receptor of the TNF family whose activation is more commonly associated with cell death is CD95 (also known as Fas/Apo-1/TNFR6). The role of aSMase in CD95 signaling has centered around the observation that capping or clustering of CD95 is defective in the absence of a functional aSMase. The first report of in vivo significance of the aSMase/ceramide pathway in CD95 ligand (CD95L)-induced cell death demonstrated that aSMase knockout mice were protected from hepatocyte apoptosis after CD95 activation in a model of autoimmune hepatitis. Requirement for ceramide has been further supported by studies using exogenous ceramide to reverse CD95-resistance in aSMase-null mice. Exogenous ceramide bypasses the metabolic block resulting from genetic absence of aSMase, demonstrating a strict requirement for ceramide in receptor-mediated cell death.

4.3. aSMase can be activated independently of extracellular receptors to regulate apoptosis

In addition to its role in death receptor-mediated cell death, the aSMase/ceramide pathway is activated by several toxic stimuli (e.g., radiation, chemotherapeutic drugs, toxic metals) that lead to ceramide-dependent cell death through cellular mechanisms that are largely unknown (see Clinical Case 9-1: aSMase and Wilson's disease). The earliest connection between defects in sphingomyelinase-mediated ceramide generation and diminished apoptosis came in 1996 when the Kolesnick lab demonstrated an in vitro and an in vivo requirement for a functional aSMase in ionizing radiation (IR)-induced apoptosis (Santana et al. 1996). IR induces

apoptosis by producing double-strand DNA breaks and can signal cell death through a p53-dependent pathway. However, human lymphoblasts from NPD patients lacking aSMase exhibit resistance to IR, and sensitivity can be restored by retroviral delivery of aSMase cDNA. In vivo protection from IR-induced apoptosis in aSMase-deficient mice is also seen in multiple tissues after whole-body irradiation; these include the lung, central nervous system, intestinal tract, and spleen. The authors also compared the apoptotic response in aSMase knockout mice with mice lacking p53. The intriguing results are that although certain tissues seem to rely on aSMase as a primary mediator of IR-induced apoptosis (e.g., microvascular endothelial cells), other tissues seem to have a lesser requirement for aSMase and a greater need for functional p53 (e.g., thymus). Thus the role and/or the regulation of aSMase may be unique to cell types that are less reliant on p53 signaling.

Besides its function in radiation-induced death of normal tissues, aSMase may represent an important mediator of radiation-induced death of cancer cells. Given the utility of IR in the treatment of various cancers, the role of aSMase in mediating IR-induced tumor regression has also been examined. IR-induced tumor regression was shown to require intact aSMase, and the effect was reported to involve IR-induced microvascular endothelial cell apoptosis. Garcia-Barros et al. (2003) demonstrated that cancer cells grew to larger overall tumor size implanted subcutaneously in aSMase-null mice as compared with wild-type mice, but more importantly, in response to IR treatment, the fibrosarcoma cells grown in aSMase-null mice did not respond, whereas tumor size was diminished in the wild-type mice. This effect was later confirmed to involve endothelial cell viability of the host mouse, although the role of primary endothelial injury in IR-induced tumor cell death remains a highly contentious issue.

4.4. Controversial aspects of the role of aSMase in apoptosis

Although these examples may be compelling, a universal role for the aSMase/ceramide pathway in cell death cannot be fully substantiated by the available evidence. For example, aSMase deficiency does not confer protection from cell death in all tissues to the same extent. Lin et al. (2000) reported that CD95 activation induced apoptosis in wild-type and *SMPDI*^{-/-} B and T lymphocytes equally well, whereas hepatocytes from *SMPDI*^{-/-} mice were markedly protected from death. In addition to differences in cell type dependence, it has been shown that protection from cell death in mouse

CLINICAL CASE STUDY 9-1: THE aSMASE/CERAMIDE PATHWAY IN WILSON'S DISEASE

Wilson's disease (WD) is an autosomal-recessive metabolic disorder resulting from mutations in *ATP7B*, a gene that encodes a P-type ATPase Cu^{2+} transporter. Defects in *ATP7B* lead to dysregulation of Cu^{2+} homeostasis, ultimately causing build-up of copper in tissues throughout the body. Over time the accumulation of copper can reach toxic levels in many tissues, including the liver, skeletal muscle, bone, nervous system, and formed elements of blood. Excessive levels of Cu^{2+} in the liver and blood result in death of hepatocytes and erythrocytes, respectively, and consequently produce hepatitis and anemia.

Lang et al. (2007) recently provided evidence for involvement of the aSMase/ceramide pathway in the induction of apoptosis in response to toxic levels of Cu^{2+} . In primary hepatocytes, Cu^{2+} overload induced a dose-dependent increase in total ceramide that was mirrored by elevations in aSMase activity. Genetic or pharmacological loss of aSMase protected hepatocytes from Cu^{2+} intoxication and cell death, demonstrating that aSMase is an important mediator of Cu^{2+} toxicity.

The authors also investigated the role of the aSMase/ceramide pathway in the pathophysiology of Cu^{2+} -induced anemia of WD. Patients with WD have elevated aSMase activity in plasma derived from leukocytes. Similarly to hepa-

tocytes, Cu^{2+} induced activation of aSMase in leukocyte cellular extracts, but importantly, Cu^{2+} also stimulated secretion of aSMase into the culture medium. Incubation of purified erythrocytes with conditioned media from Cu^{2+} -treated leukocytes induced ceramide accumulation in erythrocytes. The increases in ceramide were associated with PS externalization, a feature of programmed cell death in erythrocytes (also known as eryptosis). More importantly, conditioned media produced by leukocytes from aSMase knockout mice did not induce PS exposure and eryptosis in response to Cu^{2+} . These data support a model in which secretory aSMase is released by Cu^{2+} -overloaded leukocytes to generate ceramide on erythrocytes – ultimately leading to erythrocyte loss and anemia.

Although the upstream mechanisms of Cu^{2+} -induced activation of aSMase remain unknown, the downstream role of aSMase-derived ceramide in hepatocellular death and anemia supports a crucial role for ceramide in cell death signaling and identifies aSMase as a novel therapeutic target in the treatment of WD. *ATP7B*-deficient rats treated with the aSMase inhibitor amitriptyline experience reduced aSMase activity, liver fibrosis, and eryptosis. More significantly, aSMase inhibition confers a survival advantage to these rats compared with untreated controls. Although still in its infancy, modulation of aSMase activity may one day be a viable therapeutic strategy in the treatment of WD.

embryonic fibroblasts (MEFs) from *SMPD1*^{-/-} mice is stress-type specific. Lozano et al. (2001) demonstrated that *SMPD1*^{-/-} MEFs were markedly protected from IR but not from staurosporine induced cell death. These MEFs also exhibited intermediate protection from other forms of cell death (e.g., serum withdrawal and TNF- α). Overall, the evidence suggests that the aSMase/ceramide pathway may be “recruited” only in certain forms of cell death. Moreover, even when using a variety of aSMase-deficient Niemann-Pick cell lines, Bezombes et al. (2001) demonstrated that aSMase was dispensable for apoptosis in response to established “inducers” of aSMase, including IR and CD95L. Nix and Stofel (2000) showed that aSMase-deficient T cells were actually more susceptible to CD95L stimulation, further highlighting the varied nature of responses that have been reported. Thus, although some of these discrepant findings can be attributed to different cell lines or stimuli, clearly they cannot account for variability within specific model systems. Given the conflicting reports, the aSMase/ceramide pathway may not represent an integral component of the cell death

program, but rather a modifier of apoptotic signaling that is preferentially recruited under certain conditions. Future experimentation using gain-of-function experiments to complement loss-of-function studies, as well as the development of tools (e.g., specific inhibitors) to identify the form(s) of aSMase responsible for generating plasma membrane ceramide (i.e., secretory vs. lysosomal aSMase), will help to ascertain the signaling function of the aSMase/ceramide pathway.

4.5. De novo ceramide synthesis regulates programmed cell death

As mentioned above, SMases and SMase-dependent ceramide production can control programmed cell death in a variety of contexts. However, as research into sphingolipid signaling expanded in the 1990s, it became apparent that there are SMase-independent pathways of ceramide accumulation during cell death. Researchers discovered death-induced ceramide production in the absence of increased SM hydrolysis. Furthermore, the discovery of small-molecule inhibitors of de novo

Box 9-3. Fungal metabolites are essential tools for the study of sphingolipids

Two major fungal metabolites have become standard tools for the sphingolipid researcher. The first is myriocin, also known as ISP-1, a fungal metabolite of *Isaria sinclairii* that was discovered to have immunosuppressive effects. The structure of myriocin is highly similar to that of sphingosine (Figure B9-3B), leading researchers to find that the compound is a potent inhibitor of serine palmitoyltransferase. Since its discovery, myriocin has been used extensively to characterize sphingolipid biochemistry and sphingolipid-mediated biology. The other inhibitor that has enjoyed widespread use is fumonisin B₁ (FB₁), a member of a family of fungal toxins called fumonisins that are produced by *Fusarium moniliforme*, a fungus that infects several grain species, especially corn (maize). Like myriocin, the structure of fumonisin bears resemblance to that of sphingosine (Figure B9-3C) and was therefore tested for its effects on sphingolipid metabolism. FB₁ inhibits the acylation of dihydrosphingosine and sphingosine by CerS, thus reducing ceramide production and causing the accumulation of sphingoid bases. As with myriocin, FB₁ has been invaluable to the sphingolipid researcher and essential to the study of de novo ceramide synthesis.

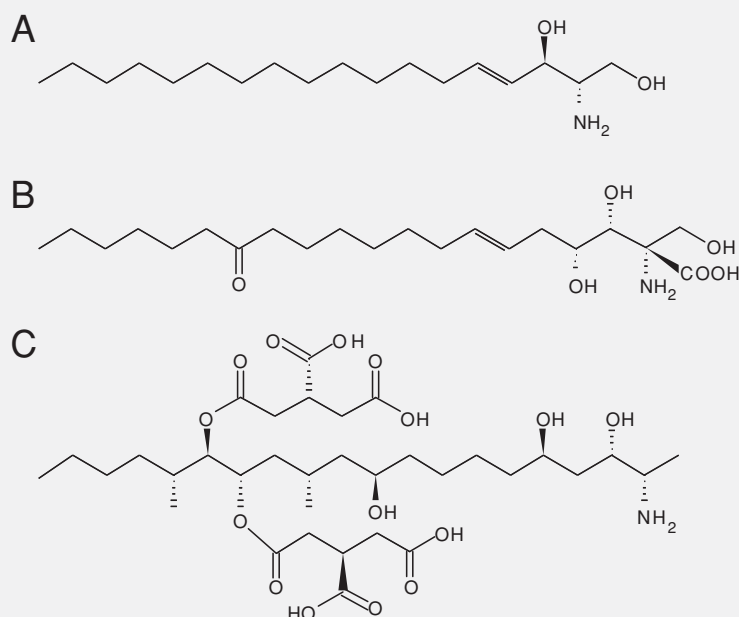


Figure B9-3. Structures of sphingosine and sphingolipid-like fungal products. (A) Sphingosine, (B) myriocin, and (C) fumonisin B₁.

sphingolipid synthesis allowed investigators to use these compounds to probe the mechanisms of ceramide-mediated apoptosis (Box 9-3).

De novo synthesis as a means of death-induced ceramide generation was first described for the chemotherapeutic agent daunorubicin in 1995. Researchers found increasing ceramide generation over the course of hours after treatment of leukemia cells with daunorubicin. The accumulation of ceramide occurred in the absence of SMase activation. Instead, CerS activity was increased, and the CerS inhibitor FB₁ was able to inhibit ceramide production and cell death. Since these preliminary studies, a myriad of proapoptotic agents have been shown to induce de novo ceramide generation in several model systems (Figure 9-7).

One system that illustrates a unique requirement for CerS and de novo ceramide synthesis is the induction of B-cell apoptosis through activation of the B-cell receptor (BcR). Treatment of B cells with anti-immunoglobulin M

causes BcR activation, ceramide production, mitochondrial dysfunction, and apoptosis. Treatment with FB₁ prevents the production of ceramide and downstream apoptotic events. Additional studies show that de novo ceramide generation participates in BcR-mediated proteasome activation and degradation of the endogenous caspase inhibitor X-linked inhibitor of apoptosis (XIAP). It is not known how ceramide directly controls these downstream effects, but the identification of targets for endogenously generated ceramide is currently an important goal in this field of research.

What enzymes are involved in de novo ceramide production during cell death? The answer to this question depends on the combination of death inducer and model system. Some studies have shown death-induced activation of SPT, whereas others provide evidence for ceramide synthase activation. For example, cannabinoids such as Δ^9 -tetrahydrocannabinol induce cell death in glioma cells that are dependent on

activation of SPT. On the other hand, irradiation of human keratinocytes with UVB light activates CerS to produce ceramide. In many cases, ceramide accumulation is shown to be dependent on *de novo* synthesis (via pharmacological inhibition), but whether activation of ceramide synthesis *per se* occurs at the enzymatic level has yet to be demonstrated for the majority of cases and demands further investigation.

4.6. p53 and Bcl-2-like proteins are connected to *de novo* ceramide synthesis

Despite being studied in numerous cell death models, the mechanisms of death-induced *de novo* ceramide generation, either by SPT or ceramide synthases, are poorly defined. One key upstream mediator that has been identified is the tumor suppressor p53, which accumulates in response to DNA damage. Treatment of Molt-4 leukemia cells with the transcriptional inhibitor dactinomycin or γ -irradiation causes p53 accumulation, ceramide production, and apoptosis. Blocking p53 by expressing the human papillomavirus protein E6 – an inhibitor of p53 – prevents ceramide accumulation and cell death. Other clues about the regulation of *de novo* ceramide synthesis come from studies of the role of the DNA damage response kinase ATM in regulating IR-induced intestinal damage. Loss of function ATM sensitizes mice to low-dose radiation and promotes ceramide synthase activation. How p53 connects the genotoxic stress response to the modulation of ceramide levels remains to be answered. However, recent studies begin to implicate sphingosine kinase (SK1) as a possible mediator of the p53 effects. Activation of p53 was shown to result in loss of SK1, possibly through proteolysis with subsequent accumulation of ceramide.

Some studies show that exogenous ceramide can promote the increase of p53 itself, resulting in the activation of a p53-dependent cell death pathway. On the other hand, exogenous ceramide can also promote cell death in the absence of functional p53. The latter evidence is exciting from a therapeutic standpoint. Unlike DNA-damaging chemotherapeutics, ceramide or ceramide analogs may be effective therapeutic adjuncts in the treatment of cancers lacking functional p53.

Members of the Bcl-2 family of proteins are critical regulators of apoptosis. Generally, they are the gatekeepers to the mitochondrial pathway of programmed cell death – a critical step in the commitment of a cell to its own demise. Bcl-2 family members, including both proapoptotic and anti-apoptotic proteins, can also control late ceramide production in ways that are not fully understood. In

some cell systems, late ceramide production occurs upstream of Bcl-2 action and the mitochondrial pathway, whereas in others ceramide production occurs downstream of these events. In MCF-7 breast adenocarcinoma cells, over-expression of either Bcl-2 or Bcl-X_L inhibits TNF- α or camptothecin-induced ceramide generation and cell death. However, in other systems (e.g., vincristine-induced death in acute lymphoblastic leukemia) ceramide generation is upstream of Bcl-2-like proteins. Along similar lines, ceramide may influence mitochondrial permeabilization, either through direct protein-lipid interactions with proapoptotic proteins such as Bax or by influencing the formation of channels in the mitochondrial outer membrane.

Several targets of *de novo*-generated ceramide have been described. *De novo* ceramide production has been linked to activation of the proteasome and degradation of the caspase inhibitor XIAP. In other studies, ceramide production was shown to specifically regulate splicing of Bcl-X and caspase-9; ceramide activates PP1 to promote dephosphorylation of the SR proteins, which regulate alternative splicing. *De novo* ceramide generation leads to the production of proapoptotic variants of Bcl-X and caspase-9 (Bcl-X_s and caspase-9b, respectively).

Another system that illustrates *de novo* ceramide-mediated death is apoptosis induced by cannabinoids. Cannabinoids induce apoptosis via binding extracellular receptors and promoting ceramide accumulation via the *de novo* pathway. In pancreatic cancer and glioma cells, the effects of ceramide appear to be mediated through the stress-associated protein p8. Although the mechanisms are still unclear, p8 connects ceramide to the ER stress-induced apoptosis via upregulation of the genes ATF-4 and TRB3. Despite these clues into the actions of ceramide, a detailed knowledge of effectors of *de novo* ceramide-mediated death remains elusive.

4.7. The role and regulation of *de novo* synthesis in ceramide-mediated cell death is poorly understood

Because of the conflicting evidence, it is currently impossible to make broad generalizations about late ceramide generation and its specific role in the regulation of proapoptotic signaling. Elucidating the detailed mechanisms of late ceramide generation – whether by *de novo* synthesis or not – remains an area of active research. Many studies have indicated that *de novo* synthesis is necessary for ceramide accumulation, but few have demonstrated bona fide activation of the enzymes of ceramide anabolism. Along similar lines, it is not known whether the degradative enzymes play a regulated role in controlling ceramide accumulation; as mentioned

CLINICAL CASE STUDY 9-2: CERAMIDE-MEDIATED EMPHYSEMA FOLLOWING BLOCKADE OF VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTORS

Pulmonary emphysema is a disease that affects millions of people throughout the world and is most commonly associated with cigarette smoking. The disorder is characterized by destruction of the alveoli of the lungs, enlargement of the airspaces, and a reduction in lung surface area. These defects ultimately lead to a loss of gas exchange and deteriorating respiratory function.

Vascular endothelial growth factor (VEGF) is a peptide growth factor that plays a key role in growth and maintenance of the endothelial cells that line the blood vessels of the body. Several studies indicate that VEGF is also necessary for survival of endothelial cells in the lung vasculature.

Treatment of mice or rats with SU5416, a VEGF receptor blocker, causes apoptosis of endothelial and alveolar cells, loss of alveolar structure, and the onset of pulmonary emphysema.

Petrache et al. found that, along with increased cell death and emphysema, VEGF blockade using SU5416 induced ceramide synthase activation, secretory aSMase activation, and ceramide production in the lungs (Petrache et al. 2005). Administration of myriocin or FB₁ blocked the increases in ceramide, decreased SU5416-induced caspase-3 activation, and prevented the loss of alveolar structures. More importantly, these data show a direct role for de novo ceramide synthesis in the pathogenesis of emphysema, offering the possibility that targeting sphingolipid biosynthesis may be a useful therapeutic strategy for the prevention and treatment of this disease.

above, the strongest evidence points to a role for SK1, although other studies suggest proapoptotic roles for sphingosine kinase 2 (SK2). Ceramide levels are also highly regulated by metabolism into SM and GSL such that inhibition or loss of GlcCer synthase or SM synthase can result in increased cellular ceramide and, in some but not all cases, increased cell death.

Investigations into the details of sphingolipid metabolism, especially through the cloning and characterization of the enzymes involved (e.g., SPT, CerS1–6, aSMase), are beginning to bear fruit in terms of understanding the function of these enzymes and their products in the cell death process. Illustrative examples of this fact are seen in the case studies examining aSMase-mediated death using knockout animals (mentioned previously). As for de novo ceramide synthesis, a handful of studies have demonstrated an *in vivo* role for CerS-dependent ceramide generation in controlling apoptosis-mediated disease (see Clinical Cases 9-2 and 9-3).

Through the use of more specific pharmacological inhibitors, manipulations of specific enzymes via overexpression and knockdown, and mice with knockouts of the enzymes of de novo synthesis, the understanding of de novo ceramide-mediated signaling and apoptosis will be greatly enhanced.

5. CONCLUDING REMARKS AND FUTURE DIRECTIONS

The previous four sections have introduced the concept of bioactive lipids and the role of sphingolipids as bioactive lipid mediators in cell death and provided specific examples of studies implicating regulated ceramide

production in the cellular program activated by inducers of cell death. In response to various inducers of cell death, ceramide is generated through either the SMase or de novo pathway – or a combination of the two (i.e., the salvage pathway) – resulting in cell death. Inhibiting ceramide generation, either through pharmacological or genetic manipulation of enzymes of sphingolipid metabolism, often but not always rescues cells from death. Lastly, restoring ceramide formation re-engages the cell's death program.

In addition to cell death, ceramide is emerging as a mediator of a variety of cellular processes (e.g., inflammation, cellular adhesion, senescence, and cell cycle arrest). It has become obvious that ceramide, although a key mediator of apoptosis, has signaling roles that are not restricted to cell death pathways. Differences in signaling pathways that connect ceramide to various biological effects likely occur in a cell type- and stimulus-specific manner. The current evidence points to ceramide signaling as a module that may be used in a variety of contexts, of which programmed cell death is only one. If this is true, then an understanding of the multiple ceramide-mediated pathways is of paramount importance to designing ceramide-based therapeutic interventions that have specificity for a particular target system (e.g., killing cancer cells vs. normal tissue).

For example, a comparison of aSMase-mediated signaling with de novo-mediated signaling raises the obvious question: “Are all ceramides created equal?” The available evidence indicates that ceramide signaling is sufficiently complex to necessitate a careful dissection of the individual pathways. For that reason, the future of research in the field of ceramide-mediated signaling

CLINICAL CASE STUDY 9-3: RADIATION-INDUCED COLONIC CELL DEATH

Ionizing radiation (e.g., x-rays and γ -rays) is used clinically to treat several forms of malignancy as well as to prepare patients for bone marrow transplant. One of the adverse consequences of treating with ionizing radiation, particularly to the abdomen, is damage to the cells of the intestine. Intestinal damage leads to inflammation, and patients experience symptoms such as nausea, vomiting, and diarrhea – a disease known as the gastrointestinal (GI) syndrome.

Using mice as a model, researchers have found that two different sphingolipid pathways regulate radiation-induced intestinal injury. One of the principle causes of injury after irradiation is loss of the vascular endothelium in the blood vessels supplying the intestine. The endothelial cells lining the blood vessels of the intestine are particularly sensitive to radiation and undergo apoptosis. Intriguingly, endothelial cells from aSMase knockout mice (*SMPD1*^{-/-}) do not

undergo apoptosis after low-dose radiation. As a result, *SMPD1*^{-/-} mice are partially protected from irradiation and have an increased survival as compared with wild-type mice.

At higher doses of radiation (>18 Gy), however, intestinal injury becomes independent of endothelial damage and is the result of direct damage to the epithelial cells lining the GI tract. At these doses, *SMPD1*^{-/-} mice are not protected compared with their wild-type counterparts. The high-dose radiation induces ceramide synthase activity, and there is a subsequent increase in ceramide production in the intestinal tissue. Researchers found that administration of FB₁ to wild-type mice could inhibit radiation-induced ceramide synthase activity, ceramide production, and epithelial cell apoptosis. More importantly, the mice survived longer. Although the mechanisms behind these effects remain to be defined, these studies illustrate the crucial but complex role sphingolipid metabolism can play in programmed cell death signaling.

and apoptosis is concerned with several issues that can be framed into several key questions, outlined as follows.

5.1. Who? (Which enzyme?)

At the expense of anthropomorphizing the enzymes of sphingolipid metabolism, it is crucial to address the question of which enzyme – “who” – is responsible for particular signaling events in the context of cell death. As elaborated previously, numerous enzymes control the balance and flux of sphingolipids both at the basal state and during drastic changes in cell function such as apoptosis. aSMase and CerS have thus far been heavily implicated in death-induced ceramide increases, but the mechanisms of their involvement are ill-defined. As discussed below, the particular enzyme involved dramatically affects the location and composition of the ceramide produced (i.e., specific chain lengths), its ability to be metabolized by additional enzymes (e.g., CDases, SM synthases), and its interaction with putative effectors (e.g., protein phosphatases, cathepsin D). Identification of the specific genes and protein products regulating ceramide production in apoptosis is a key initial step to elucidating the mechanisms of ceramide-mediated death.

5.2. What? (Which ceramide?)

With the development of more sensitive and specific techniques for analysis of the thousands of sphingolipid

species – the “sphingolipidome” (see [Box 9-2](#)) – it is becoming understood that “ceramide” does not represent a single molecule, but rather a large variety of distinct molecular species with variable acyl chain lengths, degrees of saturation, and other modifications (e.g. α -hydroxylation). An obvious question is “What functions or advantages does a particular ceramide repertoire impart?” Recent evidence suggests that acyl chain length can be controlled at the level of CerS. Mammals possess six CerS (CerS1–6) that have distinct preferences for the acyl-CoA used in the formation of ceramide. For example, CerS1 synthesizes ceramide from stearoyl- and oleoyl (C₁₈- and C_{18:1}-ceramides), whereas CerS2 synthesizes predominantly very long-chain ceramides (C₂₂- through C₂₄-ceramides). Moreover, several studies have suggested distinct roles for individual CerS and their ceramide products in regulated cell death.

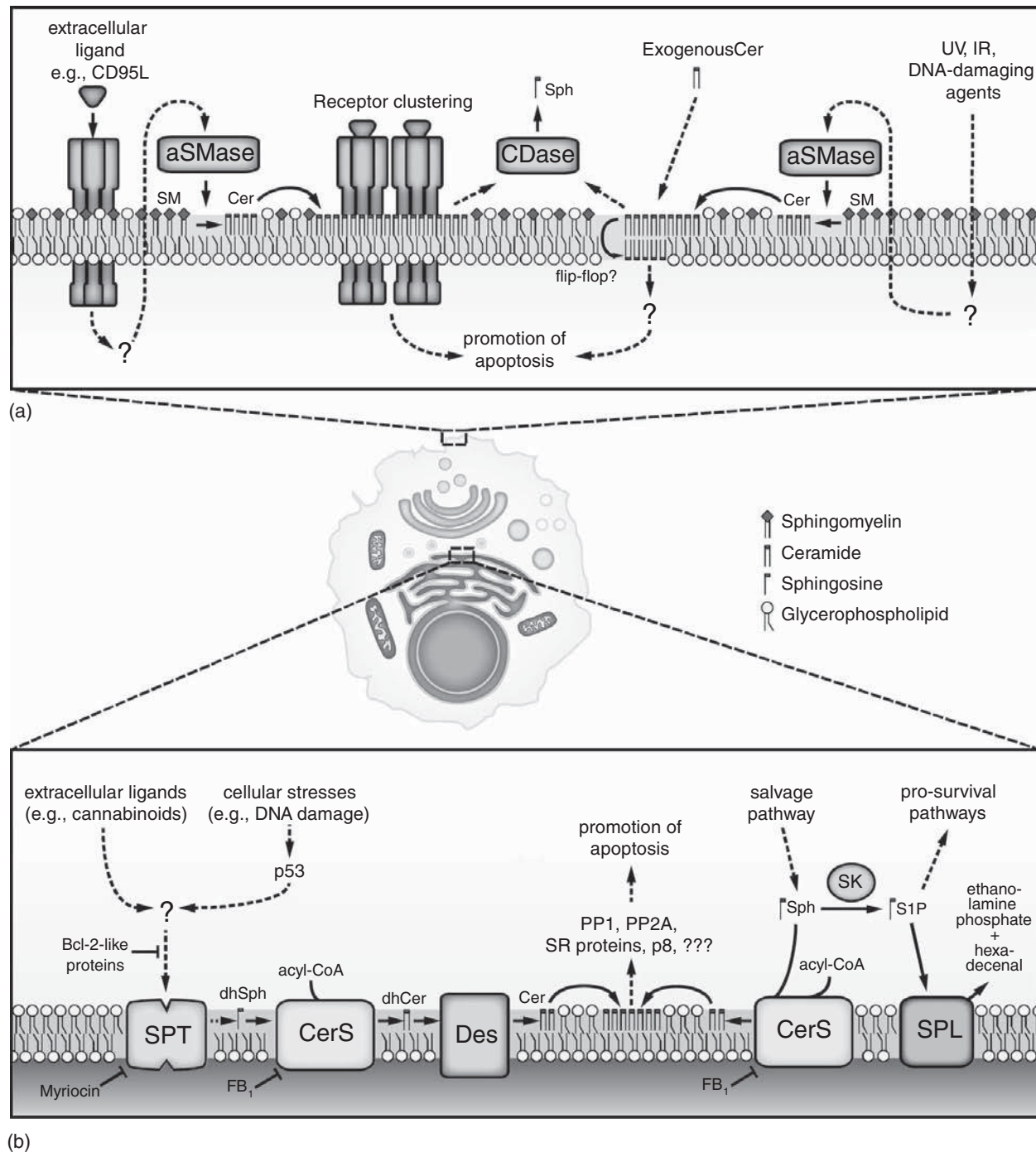
5.3. Where? (Which compartment?)

Perhaps the most important consideration in terms of the study of bioactive lipids is the question of where the lipid is located within the cell. Although some lipid mediators are soluble once released from a lipid precursor (e.g., IP₃), ceramide is an extremely hydrophobic lipid, and thus ceramide-mediated biology is likely to occur in close proximity to biological membranes. Given that sphingolipid metabolic enzymes have been detected in various subcellular regions, one can easily posit that ceramide is restricted to specific membranes

to serve specific functions (Figure 9-8). Thus SMase-derived ceramide at the plasma membrane is unlikely to serve the same signaling function as ceramide derived from upregulation of the *de novo* pathway, which localizes almost exclusively to the ER.

Additionally, the ability of ceramide to promote cell death signaling is counterbalanced by other sphingolipid enzymes that have the capacity to “detoxify” the cell of ceramide as it is generated. The ceramidases, for example, not only relieve the burden of accumulat-

ing ceramide, but also act as a shunt to generate other bioactive sphingolipids, such as sphingosine or the pro-survival lipid, sphingosine-1-phosphate. Thus a death-inducing signal can be transformed to a survival signal by virtue of other sphingolipid metabolic enzymes. Ceramide increases may also be buffered by metabolism into sphingomyelin and glycosphingolipids; in fact, the conversion of ceramide into glycosphingolipids via glucosylceramide synthase has been shown to be a mechanism of drug resistance in cancer cells.



5.4. When? (At what steps?)

The temporal relationship between the time of ceramide generation and the onset of cell death continues to be an issue of confusion and contention. Activation of aSMase is commonly seen as an acute phenomenon, separated from the first signs of cell death by several hours. On the other hand and depending on the stimulus, late ceramide accumulation occurs after a few hours of stimulation and may be coincident with some of the known downstream apoptotic mediators (e.g., mitochondrial permeabilization, caspase activation). If ceramide by itself is essential for cell death, why are there two pathways to make it? Is this a form of redundancy, or are there distinct functions for SMase-derived ceramide and *de novo* ceramide? To answer these questions, it will be imperative to use the growing knowledge of the particular enzymes – aSMase, SPT, and CerS1–6 – and their sub-cellular localization to experimentally address the contribution of each to apoptotic signaling.

5.5. How? (Through what mechanisms?)

To ask *how* is to question the mechanism by which an event occurs. In the context of ceramide-mediated cell death, there are basically two general mechanisms of interest. First, how is ceramide generated? The answer to this question most highly depends on answering the first question of which enzyme is responsible for the accumulation of ceramide. Identification of responsible enzymes allows the experimenter to use molecular tools to dissect the role of that particular enzyme in regulating apoptosis.

The second issue is that of *how* ceramide exerts its effects. There are abundant data to suggest that

ceramide functions both as a second messenger (e.g., activating protein phosphatases PP1/PP2A) and as a modulator of membrane structure (e.g., promoting microdomain formation and CD95 clustering). Unlike DAG, for which a specific protein interaction domain has been identified and characterized, the direct interaction of ceramide with candidate effector proteins through a particular protein motif has yet to be demonstrated. Many studies have implicated certain ceramide-interacting proteins in cell death (e.g., ceramide/PP2A interaction controlling Bcl-2 and Bax phosphorylation, or ceramide/cathepsin D interaction inducing activation of Bid), but robust connections between ceramide, these mediators, and the control of apoptosis remain to be delineated. The dearth of mechanistic explanations for ceramide signaling may appear surprising given the abundant data supporting roles for ceramide in mediating programmed cell death; however, the study of lipid-protein interactions remains one of the most difficult and vexing areas of biochemical research. Despite these shortcomings, investigations into the detailed mechanisms of ceramide signaling remain promising. The identification of the numerous genes governing sphingolipid metabolism as well as new tools in the detection and manipulation of sphingolipid levels are allowing unprecedented insight into the complexities of this field of research.

5.6. What purpose?

Although the “purpose” of any biological phenomenon is a matter of philosophy, one can ask what the contributions of ceramide signaling are to the evolutionarily conserved program of apoptosis. The road to apoptosis involves a vast multitude of molecular factors, of which

Figure 9-8 (facing page). Summary of ceramide-mediated pathways. (A) Activation of the aSMase/ceramide pathway has been reported in response to various inducers of cell death. The role of aSMase in both receptor-mediated and receptor-independent cell death centers on its ability to generate ceramide at the plasma membrane after stimulus-mediated re-localization. Ceramide generation and accumulation promotes apoptotic signaling through influencing microdomain formation in the plasma membrane and subsequent oligomerization of death receptors and/or acting as a lipid second messenger to various candidate effector proteins. Through either, or both, of these mechanisms, aSMase-derived ceramide promotes cell death. Requirement for the aSMase/ceramide pathway in cell death is suggested as follows: (1) pharmacological or genetic disruption of aSMase protects a variety of tissues and cells from various inducers of cell death; (2) restoration of aSMase cDNA into aSMase-null tissues, or addition of exogenous recombinant aSMase enzyme, restores ceramide generation and cell death; and (3) adding exogenous ceramide restores apoptotic signaling in aSMase-null tissues and cells supporting a role for the lipid product of aSMase action, and not the aSMase enzyme itself. (B) Ceramide may also accumulate via the stimulation of *de novo* synthesis. *De novo* ceramide synthesis occurs in the ER, where many enzymes of sphingolipid metabolism reside. The mechanisms leading to increased *de novo* synthesis are unclear, but several studies have implicated p53 and Bcl-2-like proteins as upstream regulators of this process. Increases in ceramide during cell death can occur due to enhanced activity of SPT or CerS or due to decreased metabolism into other sphingolipids. Alternatively, increases in ceramide may occur when free sphingosine increases, which may occur hypothetically via activation of the salvage pathway or when SK activity is decreased (e.g., via proteolysis). Depending on the stimulus, the downstream effects of ceramide may be mediated through activation of PP1 or PP2A, promotion of alternative mRNA splicing via SR proteins, activation of the ER stress protein p8, or as yet unidentified protein targets. See Color Plate 9.

ceramide is only one module. On the basis of current understanding, one might speculate that those events that specifically require membrane involvement (e.g., receptor signaling, lysosomal permeabilization, mitochondrial outer membrane permeabilization) are more likely to depend on ceramide signaling. This final question will only be answered when all of the previous questions are answered and the pieces of the conceptual puzzle are amassed and assembled.

6. SUMMARY

- Lipid signaling is an essential component of multiple cell signaling processes. In the context of programmed cell death, the sphingolipid ceramide is generated by multiple mechanisms to promote cell death.
- Sphingolipid metabolism is a complex and highly regulated process with ceramide being a crossroads for the generation and breakdown of multiple sphingolipid species.
- Cellular levels of ceramide and its metabolites are regulated by enzymes of sphingolipid metabolism through multiple enzymatic pathways, including de novo synthesis, sphingomyelinase-mediated sphingomyelin hydrolysis, and re-acylation of free sphingosine via the salvage pathway.
- Ceramide accumulates in cells and tissues undergoing apoptosis in response to a wide variety of stimuli.
- An increase in ceramide, whether endogenously or exogenously, induces cell death or sensitizes cells to death.
- Elimination of ceramide accumulation prevents programmed cell death in a variety of cell death models.
- aSMase-mediated ceramide production via SM hydrolysis at the plasma membrane and/or the endolysosomal system is relatively acute after stimulation and modulates receptor-mediated and non-receptor-mediated pathways.
- An increase in the de novo generation of ceramide, due to SPT and/or CerS activation, occurs after several hours of proapoptotic stimulation and may be regulated by p53 and Bcl-2 family members.

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Cytotoxic Granules House Potent Proapoptotic Toxins Critical for Antiviral Responses and Immune Homeostasis

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1. GENERAL INTRODUCTION

1.1. Cytotoxic lymphocytes and apoptosis

The immune system of high-order organisms is a highly specialized compartment that eliminates transformed cells and cells infected with viruses or bacteria through a controlled process of cell-mediated cytotoxicity. The immune cells responsible for mediating cell death are collectively called cytotoxic lymphocytes (CLs) and are made up of natural killer (NK) cells and cytotoxic T lymphocytes (CTL). CLs are distinguished primarily by their respective mechanism of antigen recognition. NK cells form part of the innate immune response, a generalized first line of defense. NK cells are generally CD3⁻CD56⁺ lymphocytes that recognize and respond to abnormal cells through an imbalance of facilitatory and inhibitory receptors (Bottino et al., 2004; Moretta et al., 2004). CTLs form part of the adaptive immune response, a more specific response that is generated subsequent to and as a consequence of the innate response. These cells use their clonotypic T-cell receptors (TcRs) to recognize a peptide antigen presented on the major histocompatibility complex (MHC) proteins on the surface of the target cell. CTLs can be identified on the basis of expression of CD3 and CD8 (CD3⁺CD8⁺) on their cell surface. In addition, some CD4⁺ T cells (typically T-helper cells) can have limited cytotoxic capacity.

Although NK cells and CTLs recognize their targets through different receptors, both can kill their targets by one of two specific and directed processes: ligation of death receptors or granule exocytosis (Cohen et al., 1985; Lowin et al., 1994). NK cells primarily use the granule exocytosis pathway, whereas CD8⁺ CTLs typically use the granule pathway to kill virus or transformed cells, although not exclusively. CD4⁺ CTLs can also use either

pathway, depending on their subtype (Table 10-1). The purpose of this chapter is to provide an outline of the granule exocytosis pathway to cell death, as receptor-mediated apoptosis is outlined in detail elsewhere.

2. CYTOTOXIC GRANULES AND GRANULE EXOCYTOSIS

The idea of a granule exocytosis pathway was first formulated after the observations of effector/target conjugates seen under the electron microscope. It was seen that on conjugate formation, cytoplasmic granules in CLs became reoriented to the area of cell:cell contact (Bykovskaja et al., 1978), and pores were then observed to form on the target cell membrane (Dourmashkin et al., 1980). These observations brought about the hypothesis that pre-stored cytotoxic proteins could be released by the CLs in a vectorial fashion toward the target cell surface after antigen recognition (Dennert and Podack, 1983). Since then, it has become clear that CLs contain unique lysosome-like compartments that directly secrete their contents toward a target cell (Burkhardt et al., 1990; Yannelli et al., 1986). These compartments have aptly been named *secretory granules* and, when purified, have been shown to exhibit both membranolysis and apoptosis in a dose-dependent manner, with no particular target cell specificity.

Similar to secretory granules (or secretory lysosomes) from other hematopoietic cells, granules in CLs contain a uniform electron dense core surrounded by a thin cortex of membrane lamellae (Burkhardt et al., 1989). In common with other lysosomes, they have a low pH and harbor typical degradative lysosomal proteins; however, secretory granules have a dual function and also house specialized proteins involved in programmed cell death, which can be secreted in a regulated fashion (Bossi and Griffiths, 2005; Peters et al., 1991; Smyth et al.,

Table 10-1. Cytotoxic mechanism used by different lymphocyte subsets

Lymphocyte subset	Cytotoxic mechanism	
	Perforin/grB	FasL/Fas
CD8 ⁺ CTL	+	+
NK	+	-
CD4 ⁺ Th1	-	+
CD4 ⁺ Th2	+	-

Source: This table is adapted from a similar table in (Trapani, 1998).

2001). The proapoptotic proteins, including perforin and granzymes, have been shown, by means of colloid gold staining, to localize to the electron dense core, possibly by association with a proteoglycan, chondroitin sulfate (Burkhardt et al., 1989; Stevens et al., 1987). The low pH provides a favorable environment for the lysosomal hydrolases and protects the CLs from the action of the proteins involved in apoptosis that require a neutral pH for optimum activity (Persechini et al., 1989; Voskoboinik et al., 2005).

To effectively kill their targets by granule exocytosis, the death-inducing proteins of the cytotoxic granules (perforin and granzymes) must be delivered from the CL into the target cell. This is a multistage process involving (1) synthesis and loading of the granule proteins into the secretory granules; (2) formation of an immunological synapse between the effector and target cell; (3) granule trafficking within the effector cell; (4) secretion of granule proteins into the immunological synapse; (5) their uptake into the target cell; and finally, (6) activation of death pathways in the target cell.

2.1. Synthesis and loading of the cytotoxic granule proteins into the secretory granules

Although both perforin and granzymes are constitutively expressed in NK cells, naive T lymphocytes do not express these cytotoxic proteins, nor are secretory granules found in their cytoplasm (Bou-Gharios et al., 1988; Olsen et al., 1990). On TcR engagement, an increase in intracellular calcium initiates signaling cascades that mediate the transcription of various lysosomal and proapoptotic proteins trafficked to the secretory granules, as well as lysosomal transmembrane proteins that help mediate cell signaling (Esser et al., 1998; Gray et al., 1987). Protein synthesis occurs within hours of TcR triggering and is accompanied by T-cell division and maturation and the appearance of secretory granules in the cytoplasm typically by 12 to 48 hours

(Bou-Gharios et al., 1988; Olsen et al., 1990; Podack and Kupfer, 1991).

Granzymes are processed like many proteases and are transported through the endoplasmic reticulum (ER) and Golgi as pre-pro proteins, where a signal peptide piece is removed. Granzymes are targeted to the lysosomes through the M6R pathway; however, an M6R-independent pathway also exists, as patients with I cell disease (a deficiency of enzyme-mediated mannose phosphorylation) still have active granzymes in their secretory granules (Griffiths and Isaaz, 1993; Masson et al., 1990). Within the secretory granules, an N-terminal acidic activation di-peptide is removed by DPP1/cathepsin C (Pham et al., 1996). The lymphocytes of cathepsin C-null mice have therefore been proposed to totally lack granzyme B (grB) activity and perforin-dependent cytotoxicity (Pham and Ley, 1999). Surprisingly however, cells targeted by allogeneic CD8⁺ CTL raised in cathepsin C-null mice can still die through perforin and grB-dependent apoptosis, albeit at a reduced rate (Sutton et al., 2007). Thus at least one other granule protease is capable of processing pro-grB.

Perforin is initially synthesized in the ER, and after cleavage of a 21-amino acid pro-piece, an intermediate form of perforin is glycosylated with complex glycan in the Golgi. In the secretory granules, an unknown cysteine protease is thought to cleave approximately 20 amino acids at the C-terminus (with the attached glycan), resulting in acquisition of lytic activity (Uellner et al., 1997). Although granzymes and perforin are stored in their active form in the secretory granules, their enzymatic function is restricted by the acidic pH of the granules, providing protection for the CL. Thus, once a CL becomes conjugated with a target cell, it is able to induce death almost immediately, because perforin and granzymes are active once they encounter the neutral extracellular pH.

2.2. The immunological synapse

Once CTL differentiation/activation has occurred, T cells recognize/interact with their targets by TcR engagement of antigen presented on MHC and form a tight seal between the effector and target cell. This junction is known as the immunological synapse (IS) (Stinchcombe and Griffiths, 2003). A mature synapse contains a specific outer ring of membrane-bound proteins mediating cell-cell adhesion and enclosing other proteins involved in signaling cascades required for protein synthesis and cell activation (Monks et al., 1998). This tight seal may prevent leakage of the cytotoxic granule proteins and facilitate their vectorial delivery to the target cell. The IS also

contains a unique secretory domain, which is the region within which secretory granules exocytose their cytolytic proteins (Stinchcombe et al., 2001). Although granules are maintained at an acidic pH, the environment in the IS has not been formally characterized (with respect to the pH and calcium concentration). It therefore remains unclear precisely how the environment of the IS supports the function of granule proteins secreted into this domain (Stinchcombe and Griffiths, 2007).

2.3. Secretion of granule proteins

CLs do not exocytose their granules randomly; granules are directed specifically to the IS. Further, in an activated CTL, the IS can form within minutes of TcR stimulation (Grakoui et al., 1999; Stinchcombe et al., 2001), and only transient interaction with target cells may take place. The process of granule redistribution from the posterior to the leading edge of an effector cell (polarization) and their exocytosis is therefore a rapid, directed, and coordinated process (Kupfer and Dennert, 1984; Yannelli et al., 1986). It is unclear how many granules are released into the synapse, although it has been suggested that not all the granules are exocytosed, with some remaining in the effector cell to allow for the serial killing often seen with an individual CL (Stinchcombe et al., 2001).

Before secretory granule movement, the microtubule organizing center (MTOC) and Golgi compartment are redeployed to the point of contact between the two cells (Kupfer and Dennert, 1984; Kupfer et al., 1985). Secretory granules cluster around the MTOC by moving along microtubules by means of kinesin- and dynein-based motors (Kamal and Goldstein, 2000). From the MTOC, secretory granules dock at the plasma membrane, fuse, and release their cytolytic proteins into the IS (Stinchcombe et al., 2004). More recently, granules have been shown to be delivered directly to the plasma membrane, a process that is believed to be dependent on centrosome placement at the plasma membrane, in particular at the central supramolecular activation cluster of the IS (Stinchcombe et al., 2006).

The various proteins involved in secretory granule migration, membrane docking, fusion, and subsequent secretion have been identified by examining the genetic defects underlying patients suffering from diseases of the secretory granules, such as Chediak-Higashi syndrome, Griscelli syndrome, Hermansky-Pudlack syndrome, and familial hemophagocytic lymphohistiocytosis (FHL) as is reviewed by Stinchcombe et al. (2004). Proteins such as Lyst, Rab27a, Munc13–4, and syntaxin 11 have all been identified to play a role in efficient

secretory granule transport to the IS (Menager et al., 2007; Stinchcombe and Griffiths, 2007).

2.4. Uptake of proapoptotic proteins into the target cell

Once released into the IS, cytolytic molecules must make their way into the target cell, and the mechanism by which this occurs remains one of the most controversial areas of granule-mediated killing. Originally, electron microscopy analysis of a killer/target conjugate showed close association of cytotoxic granules with the target membrane, suggesting that the granule contents of CL could be directly responsible for forming transmembrane channels (Dennert and Podack, 1983). Purified granules from CL showed a very high hemolytic and tumoricidal activity in the presence of calcium at 37°C and at neutral pH, compared with whole intact cells from which granules were derived (Criado et al., 1985; Podack and Konigsberg, 1984), and the cytolytic activity in these purified granules was eventually attributed to the 66-kDa protein, perforin (Masson and Tschopp, 1985). Generation of perforin-deficient mice confirmed the essential role for perforin in granule-mediated cell death (Kagi et al., 1994).

Purification of perforin revealed that it could polymerize and insert in lipid bilayers (Masson and Tschopp, 1985; Podack et al., 1985; Young et al., 1986), making pores with an internal diameter of approximately 160 Å (16 nm). It was thus proposed that the perforin pore could trigger lysis by disrupting osmotic homeostasis or stimulate a calcium-regulated processes of internal disintegration by altering intracellular calcium flux (Duke et al., 1989; Kraut et al., 1990). However, various lines of evidence suggested that CL-induced death was distinct from perforin lysis. CL-induced death was shown to involve fragmentation of DNA into oligonucleosomal-sized fragments, by a process that was explicitly dependent on granzymes, in particular grB (Shi et al., 1992). Importantly however, granzyme-dependent cell death is not evident unless perforin is present (Hayes et al., 1989; Shiver and Henkart, 1991). Therefore, perforin must function as a vehicle for the efficient delivery of granzymes into the apoptotic pathways of the target cell.

Originally, it was believed that the perforin pore acted simply as a conduit for granzyme diffusion into the cell; however, more recent studies have indicated a more complex process (Keefe et al., 2005; Trapani et al., 1998b). First, it was shown that grB could enter the target cell in an energy-dependent process without the need for perforin, but remained compartmentalized in endosomes and did not kill the target cell (Froelich

et al., 1996b; Shi et al., 1997). The mannose phosphate receptor (MPR) was thought to constitute the main pathway for granzyme entry into the cytoplasm (Motyka et al., 2000). However, more recent work has demonstrated that free granzymes do not require MPR to enter the target cell (Trapani et al., 2003). It has also been shown that a maximum of only 20% of grB is mannose-6 phosphorylated, indicating that MPR-independent pathways must exist (Bird et al., 2005). Other studies have suggested that grB is predominantly complexed with the proteoglycan, serglycin, and that the entire complex is taken up into endosomes of the target cell via the MPR pathway (Veugelers et al., 2004). Although still controversial, recent studies have provided evidence against this idea (Bird et al., 2005; Raja et al., 2005), and cells deficient in M6R expression were as efficiently killed by CLs through granzyme-mediated apoptosis as M6R expressing cells, indicating that whether complexed to serglycin or not, granzymes do not require the M6R to mediate target cell entry (Trapani et al., 2003). In the absence of receptor-mediated endocytosis, granzymes have been postulated to enter the cell through fluid-phase endocytosis (Bird et al., 2005; Trapani et al., 2003). After internalization, grB remains trapped in endocytic vesicles and cannot exert its apoptotic effects unless perforin or another pore-forming toxin is also present (Browne et al., 1999; Froelich et al., 1996b).

More recently, a direct role for perforin in grB entry into the target cell was proposed. Sublytic perforin levels caused calcium influx into the target cell, which triggered membrane repair and coincided with the endocytosis of granzymes (Keefe et al., 2005). These results suggest a requirement for the synchronous application of both perforin and grB to mediate target cell apoptosis. This proposed “endosomolytic” function of perforin, although popular as a hypothesis, has as yet not been supported by significant evidence. The different mechanisms by which perforin may facilitate granzyme entry into the target cell are shown in Figure 10-1.

2.5. Activation of death pathways by granzymes

Once released inside the target cell, granzymes are capable of processing various intracellular substrates, resulting in cell death. Granzymes are serine proteases that belong to the chymotrypsin superfamily and share common characteristics with chymotrypsin-like enzymes (Henkart et al., 1987). One of the key features of serine proteases is a triad of conserved residues (histidine, aspartic acid, and serine) at their catalytic site (Kraut, 1977; Murphy et al., 1988). A total of 11 granzymes have been identified in mice (A-G, K, L, M, and N), but only 5

Table 10-2. Chromosomal localization of functional granzyme gene subsets

Chromosomal location	Species	Granzyme(s)
“Trypsase” locus		
5q11-q12	Human	A and K
13D	Mouse	A and K
“Chymase” locus		
14q11-q12	Human	B and H
14D	Mouse	B, C, D, E, F, G, L, and N
“Metase” locus		
19p13.3	Human	M
10q21.2	Mouse	M

Note: The granzyme genes are distributed to three loci, with each subfamily constituting a broad type of substrate specificity, either trypsin-like (tryptase), chymotrypsin-like (chymase), or cleavage after methionine (metase) activity.

Source: This table is a modified version of (Trapani, 2001) and (Grossman et al., 2003).

exist in humans (A, B, H, K, and M). Granzymes in both humans and mice are grouped functionally and genetically on the basis of their genes, localizing to one of three chromosomal loci, as summarized in Table 10-2.

Granzymes have very specific substrate specificities and are clearly processing (nondegradative) enzymes. Some granzymes (grA, K) cleave at basic residues (lys, arg) and others at bulky nonpolar residues (phe, trp) and therefore have trypsin-like (“tryptase”) or chymotrypsin-like (“chymase”) activity, respectively. Similarly, specificity for asp residues (grB) or met residues (grM) results in “aspase” and “metase” activity, respectively. The disparate substrate specificity suggests that granzymes may trigger specific death pathways and/or possess quite different additional functions. A key emphasis in this chapter is to describe the biological substrate preferences of different granzymes directly resulting in cell death.

3. GRANULE-BOUND CYTOTOXIC PROTEINS

The components of the secretory granules present in the CL can be categorized according to their proposed functions (summarized in Table 10-3). Some of the granule components are discussed in greater detail below.

3.1. Perforin

As described above, perforin plays a critical role in CL biology, primarily through its ability to form a pore in lipid membranes. However, very little is known about the mechanism of perforin pore formation and the specific perforin domains involved in this process. This is due, at least in part, to difficulty in expressing significant

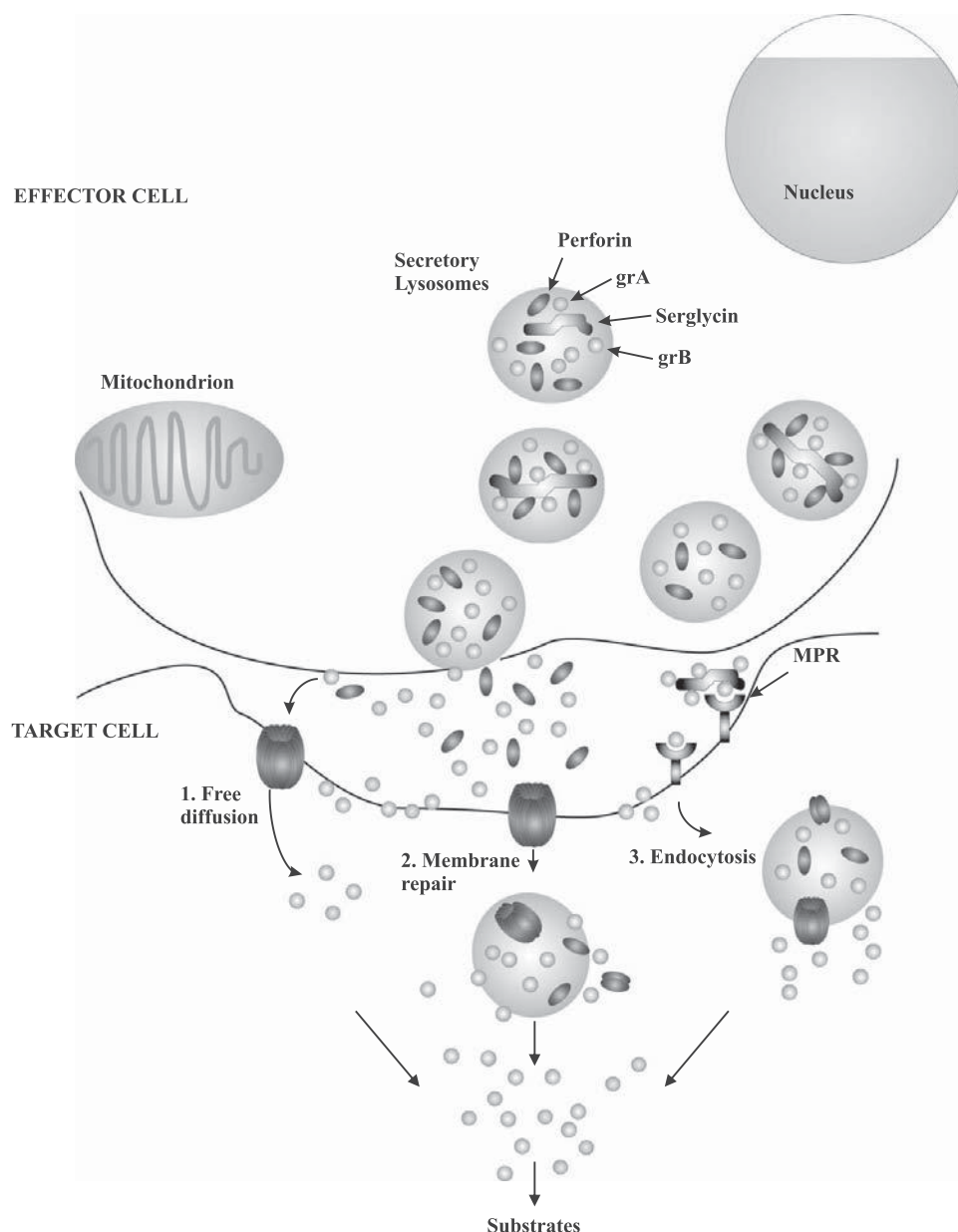


Figure 10-1. Several hypotheses have been proposed to explain how granzymes enter the target cell to mediate their cell death functions. Originally, a perforin pore was proposed to act as a conduit for granzyme entry (1). Other experiments suggest that soluble granzymes or granzymes complexed with serglycin can enter the target cell via endocytosis through the M6R or alternative generic pathways (3). More recently, perforin-mediated membrane damage has been proposed to trigger a membrane repair mechanism, which allows granzymes entry into the target cell via endocytosis (2). See Color Plate 10.

quantities of perforin for structural studies. No structure exists for perforin, and few assays that probe structure/function relationships have been devised. Human perforin is a 67-kDa protein and consists of 555 amino acids, including its 21-residue signal peptide. There are some predicted functional domains interspersed throughout the protein, which are based on a combination of direct comparisons with complement proteins, perforin peptide experiments, and more recent perforin mutagenesis studies.

The membrane-interacting domain of both complement and perforin is commonly referred to as the membrane attack complex/perforin domain (MACPF) because of sequence similarity and proposed functional homology (Kwon et al., 1989; Lowrey et al., 1989; Shinkai et al., 1988). Crystal structures of the first proteins containing a MACPF domain, human C8 α (Hadders et al., 2007; Slade et al., 2008) and Plu-MACPF (a putative toxin synthesized by the bacterium *Photorhabdus luminescens*), have recently been solved (Rosado

Table 10-3. Protein constituents of the secretory granules of CL

Granule components	Putative function	Reference
Specialized function in cell death		
Perforin	Pore formation, disruption of plasma membranes, and mediator of granzyme entry into target cell	(Masson and Tschopp, 1985; Podack et al., 1985)
Granzymes	Serine proteases with various substrate specificities. Involved in caspase-dependent and independent cell death.	(Masson et al., 1986; Masson and Tschopp, 1987; Pasternack et al., 1986)
Granulysin (human only)	Microbicidal agent. Disruption of eukaryotic and prokaryotic membranes and promoter of mitochondria-mediated apoptosis.	(Jongstra et al., 1987)
Lysosomal hydrolases		
H ⁺ ATPase	Granule acidification	(Kataoka et al., 1994)
Cathepsins B & D	Lysosomal cysteine proteases	(Burkhardt et al., 1990; Peters et al., 1991)
Cathepsin C (dipeptidylpeptidase I)	Activation of granzymes by cleavage of N-terminal dipeptide	(McGuire et al., 1993)
α -glucosidase	Lysosomal enzyme	(Burkhardt et al., 1990)
arylsulphatase	Lysosomal enzyme	(Hargrove et al., 1993; Tschopp and Nabholz, 1990)
β -glucuronidase	Lysosomal enzyme	(Orye et al., 1984)
β -hexosamidase	Lysosomal enzyme	(Tschopp and Nabholz, 1990)
Lysosomal membrane component		
FasL	Death receptor-mediated apoptosis	(Kojima et al., 2002)
CD63	Costimulatory element promoting sustained T-cell activation and expansion	(Peters et al., 1991)
Lamp-1 & Lamp-2	Lysosomal membrane proteins	(Peters et al., 1991)
Mannose-6-phosphate receptor	Granzyme trafficking within the CL	(Burkhardt et al., 1990)
Structural component		
Proteoglycan (chondroitin sulfate A)	Large negatively charged storage and carrier molecule for basic proteins	(MacDermott et al., 1985; Stevens et al., 1989; Stevens et al., 1987)
Calreticulin	Calcium binding and chaperone protein of the ER. Role in conjugate formation between effector and target cells.	(Dupuis et al., 1993)
Other		
TIA-1 and TIAR	mRNA binding, stress monitor	(Anderson et al., 1990; Kedersha et al., 1999)
Leukophysin	Granule trafficking	(Abdelhaleem et al., 1996)

et al., 2007; Rosado et al., 2008). Crucially, the structural data revealed homology with cholesterol-dependent cytolysins (CDCs), a large family of bacterial pore-forming toxins whose molecular mechanism is better understood. CDCs do not possess alpha helices capable of membrane spanning, but instead are thought to form a “pre-pore” before insertion to enable membrane-interacting domains to be revealed (Dang et al., 2005; Tilley et al., 2005). This mechanism of pore formation sees polymerization occurring after membrane binding and before membrane insertion. Based on the high degree of structural similarity predicted between perforin and these toxins, further studies are warranted to

determine whether perforin also inserts into membranes via a similar mechanism.

3.2. Granulysin

Granulysin is a cytolytic member of the saposin-like family of lipid-binding proteins (Clayberger and Krensky, 2003; Munford et al., 1995). Mice do not have a granulysin gene, and this cationic molecule is only present in the secretory granules of human CLs. It has lytic activity against various microbes, including Gram-positive and -negative bacteria, fungi, and parasites (Pena and Krensky, 1997). Granulysin has also been speculated to

contribute to tumor immune surveillance by lysing tumor cells (Kishi et al., 2002; Sekiya et al., 2002; Stenger et al., 1998; Wang et al., 2000). The mechanism of granulysin action is unclear, but it is believed to mediate its effect by association with negatively charged lipids after granule exocytosis (Kaspar et al., 2001; Krensky, 2000). The crystal structure of granulysin shows a multi-helical structure, and the association of granulysin with the plasma membrane predicts a scissoring motion, tunneling granulysin into the plasma membrane and resulting in membrane tearing (Anderson et al., 2003). Granulysin-induced membrane damage leads directly to the activation of apoptotic machinery within the cell through intracellular calcium flux and subsequent mitochondrial damage (Kaspar et al., 2001; Okada et al., 2003). Damaged mitochondria release cytochrome c and apoptosis-inducing factor, resulting in the activation of caspases and endonucleases (Kaspar et al., 2001; Pardo et al., 2001). It has been proposed that although granulysin can function independently on extracellular pathogens, it requires perforin to kill cells that harbor an intracellular pathogen (Dieli et al., 2001; Walch et al., 2007). In addition to its lytic role, granulysin can also function by recruiting immune cells to a site of inflammation (Deng et al., 2005).

3.3. Granzymes

Apart from perforin, granzymes represent the most abundant constituents of CL granules, and their role in cytotoxicity was proposed after certain protease inhibitors were shown to abrogate cytotoxicity *in vitro* (Chang and Eisen, 1980; Masson and Tschopp, 1987; Redelman and Hudig, 1980). Granzyme involvement in the apoptotic pathway of dying cells was subsequently confirmed when proteins corresponding to grA and grB were isolated from rat NK cells and displayed DNA-fragmenting ability (Shi et al., 1992).

3.3.1. GrB-mediated apoptosis

In the early 1990s, caspases were emerging as proteases that orchestrated cell death by apoptosis. Caspases are present in the cytoplasm of cells; however, they must be activated by cleavage after specific aspartic acid residues. Because grB has caspase activity, it was postulated that grB may cleave and activate pro-caspases. Indeed, using cytosolic extracts, several groups demonstrated that grB efficiently cleaved caspase-3, -7, and -8 (Fernandes-Alnemri et al., 1996; Martin et al., 1996). Caspases also cleave their substrates after specific aspartic acid residues, permitting them to autoactivate.

GrB-mediated processing of caspase-3 was partly inhibited by the addition of the caspase inhibitor zVAD-fmk, suggesting that this event occurred by a two-step process in which grB was only required for the first step (Darmon et al., 1995; Sutton et al., 2000). Subsequently, it was shown that although grB can initiate caspase activation in intact cells, even high concentrations of grB could not fully process pro-caspases on its own (Barry et al., 2000; Sedelies et al., 2008; Sutton et al., 2000; Sutton et al., 2003; Waterhouse et al., 2006a).

Importantly, caspase inhibitors could block the nuclear damage associated with apoptosis, but they could not block grB-induced cell death (Sarin et al., 1998; Trapani et al., 1998a). In contrast, over-expression of Bcl-2 in the target cell did block cell death mediated, in particular, by human grB, leading to clonogenic survival (Davis et al., 2000; Heibein et al., 2000; Sutton et al., 2000; Sutton et al., 1997). The proapoptotic BH3-only Bcl-2 family member Bid is an excellent substrate for human grB, and the cleaved product, truncated Bid (tBid), translocates to the mitochondrial outer membrane, where it can interact with Bcl-2 to release its hold on the proapoptotic Bax and Bak proteins (Alimonti et al., 2001; Heibein et al., 2000; Sutton et al., 2000). Bid's involvement in cell death mediated by human grB is crucial, as Bid-deficient cells were resistant to grB-mediated apoptosis and continued to proliferate in long-term survival assays (Waterhouse et al., 2005). After Bid cleavage, grB-mediated mitochondrial outer membrane permeabilization results in the release of proapoptotic proteins such as cytochrome c, Smac/DIABLO, and Htra2/omi (Alimonti et al., 2001; Barry et al., 2000; Sutton et al., 2000; Sutton et al., 2003). GrB has also been shown to cleave antiapoptotic Bcl2 family member Mcl-1, resulting in the release of proapoptotic Bim and subsequent cell cytotoxicity (Han et al., 2005) by a similar mechanism to that of Bid.

GrB has also been proposed to directly cleave various additional substrates that influence cell death, including inhibitor of caspase-activated DNase (ICAD), and ICAD-deficient murine embryonic fibroblasts were markedly resistant to grB-mediated DNA fragmentation (Cullen et al., 2007; Sharif-Askari et al., 2001; Thomas et al., 2000). Moreover, other nuclear substrates, such as poly(ADP-ribose) polymerase (PARP), DNA-dependent protein kinase, NuMA, and lamin B, were also directly cleaved by grB and may contribute to cell death (Andrade et al., 1998; Froelich et al., 1996a; Zhang et al., 2001a). Recent studies using human grB and primary NK cells have shown that over-expression of Bcl-2 and blocking caspase activity maintains the clonogenic survival

of target cells, suggesting that if human grB does cleave these other substrates, they do overtly result in cell death (Sedelies et al., 2008).

Many studies previously detailed strongly suggested that mitochondrial disruption precedes caspase activation during grB-mediated cell death; however, studies in mouse models suggested that mitochondria were not critical for grB-induced death (Metkar et al., 2003). Recently, the reason for these discrepancies has become clear: Although both require Asp at the P1 substrate position, human and mouse grB show differences in their substrate specificities, with human grB preferentially cleaving Bid and mouse grB being relatively more efficient at cleaving pro-caspase 3 directly (Casciola-Rosen et al., 2007; Cullen et al., 2007; Kaiserman et al., 2006). Although these distinct differences exist at a biochemical level, similar to the human system (Sedelies et al., 2008), mouse grB delivered by CTL efficiently triggered both cytochrome c release and mitochondria-independent activation of caspases (Pardo et al., 2008). In contrast to the human system, blocking mitochondrial damage and caspase activity did not protect target cells from death induced by mouse CTLs, suggesting that mouse grB may also target additional death-inducing substrates. The recent finding that the grB gene is highly polymorphic in wild mice may also provide a rationale for the different substrate specificities of human and mouse grB (Thia and Trapani, 2007). It has been proposed that viral pathogens of different species may have applied different selective pressures so that the grB gene in mice may have evolved to counter potential viral escape (Thia and Trapani, 2007). Thus the cell death pathways mediated by grB of different species may be significantly different.

3.3.2. GrA-mediated cell death

Granzyme A (grA)-induced cell death is entirely dependent on the contribution of the mitochondria and not on caspases (Beresford et al., 1999; Martinvalet et al., 2005). GrA induces loss of mitochondrial inner membrane potential and increased production of reactive oxygen species (ROS); however, the proapoptotic mitochondrial factors that are released in response to grB (cytochrome c, Smac/DIABLO, Htra2/omi) remain sequestered in the mitochondria (Martinvalet et al., 2005). ROS production is believed to then mediate the translocation of the SET complex to the nucleus, consistent with its involvement in DNA repair in response to oxidative stress (Chowdhury et al., 2006; Fan et al., 2003b). GrA has specificity for the DNA repair proteins of the SET complex, namely HMG2, Ape1, and SET (Beresford et al., 2001; Fan

et al., 2002; Fan et al., 2003b). GrA breaks the association between SET and HH23-H1, allowing HH23-H1 to act as the grA-activated DNase and nick DNA (Fan et al., 2003a). After its release into the cytosol, grA translocates to the nucleus, where it can target proteins involved in maintaining chromatin and nuclear envelop stability, such as histones, laminins, PARP, and Ku70 (Jans et al., 1998; Zhang et al., 2001a; Zhang et al., 2001b). Until recently, it was unclear how grA stimulated ROS production; however, a recent study proposes that grA enters the mitochondria and cleaves, NDUF3, a subunit of complex I in the electron transport chain (Martinvalet et al., 2008). This disrupts complex I driven respiration, resulting in increased ROS production. This, however, leaves at least two unanswered questions: (1) How does grA enter the mitochondria? (2) How does the ROS produced result in caspase-independent cell death, especially because blocking complex I with rotenone triggers cell death by a caspase-dependent mechanism (Li et al., 2003)? It is possible that ROS contributes to grA-induced death, but this is unlikely to be the whole story, and other substrates for grA remain to be uncovered. The pathways currently proposed for grA- and grB-induced cell death are shown in Figure 10-2.

3.3.3. Orphan granzyme-mediated cell death

Granzymes C, D, E, F, G, H, K, M, and N have been termed the *orphan granzymes* because their specific substrates and functions are yet to be discovered (Grossman et al., 2003). The development of the grB gene-disrupted mice resulted in the accidental knock-down of additional genes within the locus, in particular grC and grF (Pham et al., 1996). These mice have been interpreted to represent a compound knockout for several granzymes; however, grC mRNA levels were only 10-fold less than wild-type mice, when CTLs from grA^{-/-}grB^{-/-} mice were activated in mixed lymphocyte reactions. It is not clear whether this reduction was sufficient to abrogate the function of grC; however, when grB alone was “knocked down,” the cytotoxic defects were not as pronounced, suggesting a role for these orphan granzymes in cytotoxicity (Revell et al., 2005). Individual knockout models have not yet been generated for all the granzymes but will be needed to understand the individual functions of the orphan granzymes.

Purified granzymes C, K, H, and M have all been shown to be capable of inducing cell death when delivered by perforin in vitro, but their cellular substrates are unclear (Fellows et al., 2007; Johnson et al., 2003; Kelly et al., 2004; MacDonald et al., 1999). Both granzyme C and K can induce death independently of caspases,

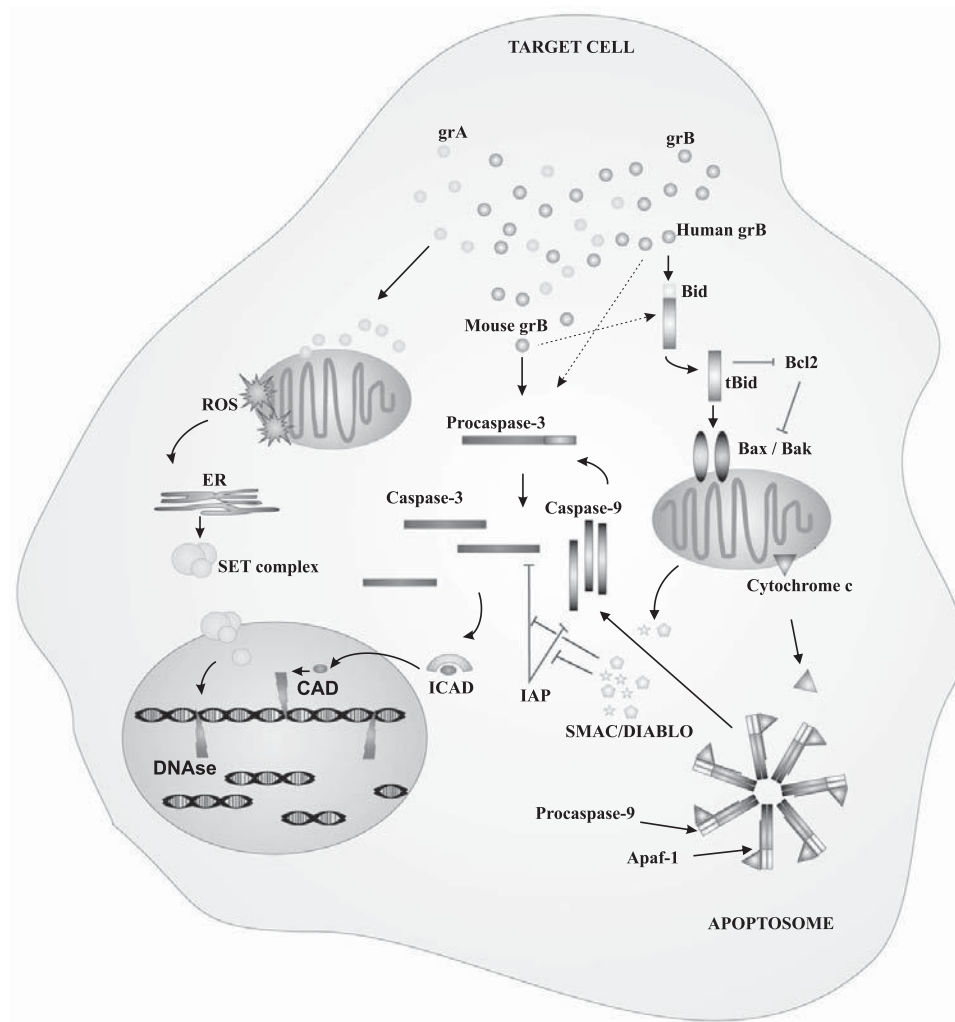


Figure 10-2. GrA and grB show different substrate specificities within the target cell. GrA induces the release of ROS from the mitochondrial inner membrane, which mediates the translocation of the SET complex from the ER to the nucleus. A DNase component of SET mediates DNA damage and subsequent cell death. Human grB induces apoptosis by cleaving Bid to tBid, where it releases Bcl-2's hold on Bax/Bak. Bax/Bak polymerize and induce mitochondrial outer membrane permeabilization, releasing mitochondrial proteins, cytochrome c, and Smac/DIABLO. Cytochrome c interacts with APAF-1 to form an apoptosome, which functions by concentrating and activating caspase-9. Caspase-9 cleaves and activates effector caspase-3, which mediates DNA damage. Smac/DIABLO function by deregulating inhibitors of apoptosis (IAP). By contrast, mouse grB preferentially induces apoptosis by directly cleaving pro-caspase-3 to active caspase-3. See Color Plate 11.

possibly through ROS production (Johnson et al., 2003; MacDonald et al., 1999; Zhao et al., 2007a; Zhao et al., 2007b). Granzyme C induces single-stranded DNA nicks, but the DNase responsible is unknown (Johnson et al., 2003). Granzyme H and K can cleave Bid; however, the kinetics are slow. Granzyme M-induced death is rapid and occurs independently of caspases and mitochondrial perturbation by direct cleavage of nuclear substrates ICAD and PARP (Kelly et al., 2004; Lu et al., 2006). The putative cell death mechanisms activated by the various granzymes are described briefly (Table 10-4).

4. A ROLE FOR GRANULE PROTEINS IN VIRAL RESPONSE, IMMUNE SURVEILLANCE, AND IMMUNE HOMEOSTASIS

It is imperative for the immune system to mount an attack on virus-infected or transformed cells, a process that is extremely dependent on cytotoxic granule proteins. It is also extremely important that the immune system diminishes lymphocyte populations afterwards to maintain cellular homeostasis. Although this latter process is thought to be primarily dependent on receptor-mediated death, cytotoxic granule proteins can also play a role, as indicated from studies with gene-disrupted mice and humans.

Table 10-4. Granzymes and their putative role in cell death pathways

Granzyme	Function
A	Mitochondrial depolarization and ROS formation; cleavage of SET complex of the ER and resulting in single-strand DNA nicks (see text)
B	Caspase-dependent and -independent apoptosis (see text)
C	Caspase-independent mitochondrial inner membrane depolarization, with subsequent ROS formation, resulting in single-strand DNA nicks (Johnson et al., 2003)
D	None yet proposed
E	None yet proposed
F	None yet proposed
G	None yet proposed
H	Caspase-independent mitochondrial inner membrane depolarization, with subsequent ROS formation, resulting in single-strand DNA nicks (also cleaves adenoviral proteins important for viral DNA replication) (Andrade et al., 2007; Fellows et al., 2007).
J	None yet proposed
K	Caspase-dependent Bid cleavage; caspase-independent mitochondrial inner membrane depolarization, with subsequent ROS formation, resulting in single-strand DNA nicks (MacDonald et al., 1999; Zhao et al., 2007a; Zhao et al., 2007b)
L	None yet proposed
M	Caspase-independent direct ICAD and PARP cleavage (Kelly et al., 2004; Lu et al., 2006; Pao et al., 2005)
N	None yet proposed

CL from perforin-deficient mice showed a marked decrease in their cytolytic function against lymphocytic choriomeningitis (LCMV) infected cells in vitro (Kagi et al., 1994). In addition to controlling the spread of viruses, there is evidence that perforin has a role in immune surveillance, that is, the elimination of transformed cells before their presentation as a clinical malignancy (Smyth et al., 2000; van den Broek et al., 1996). Considerable evidence suggests an additional role for perforin in immune regulation. Perforin-deficient mice show increased clonal expansion and persistence of virus-specific T cells and an inability to downregulate T-cell responses during chronic LCMV infection (Kagi et al., 1999; Matloubian et al., 1999). In LCMV infection, high levels of activated CTLs cannot be cleared, and the activated lymphocytes and macrophages infiltrate various organs, resulting in massive release of inflammatory cytokines, tissue necrosis, and organ failure, features very similar to those seen with human patients suffering from FHL (Arico, 1991). Among other causes, FHL can result from the absence of perforin expression within the granules and subsequent defective CL function (Stepp et al., 1999; Voskoboinik et al., 2006). Incomplete loss of perforin function has also been linked to hematological cancer, although these studies involved small numbers of patients (Clementi et al., 2005; Mehta et al., 2006; Voskoboinik et al., 2007). Perforin may also be a

critical mediator of tissue damage, controlling autoimmune beta-cell destruction that results in type 1 diabetes in nonobese diabetic mice (Kagi et al., 1997).

GrA- and grB-deficient mice both show some increased mortality when infected with ectromelia virus. However, in contrast with CTLs from grA mice whose apoptotic response is unaltered, delayed nuclear apoptotic changes are evident in target cells when treated with grB-deficient CTL (Ebnet et al., 1995; Heusel et al., 1994). In contrast to mice deficient in a single granzyme, mice that are deficient for both grA and grB are remarkably (about a million-fold) more susceptible to fatal ectromelia infection (Mullbacher et al., 1999). Furthermore, allogeneic CTLs isolated from these mice induce an alternative form of cell death that largely resembles apoptosis morphologically but features the delayed expression of markers of phagocytosis (Waterhouse et al., 2006b).

5. CONCLUSIONS

Many unanswered questions still remain regarding the function of CL secretory granule proteins. As a crucial molecule in the granule exocytosis pathway, it is unclear how perforin functions at the molecular level. Perforin is critical for mediating granzyme entry into the target cell; however, the mechanism for this synergy still remains

mysterious. Perforin's ability to form a pore seems to be necessary; however, the molecular mechanism for polymerization and membrane insertion are also unknown. Much is still unclear regarding granzyme function during granule-mediated cell death. Although it is clear that grB and grA are important contributors to granule-mediated cell death, work is currently underway to elucidate the specific contributions of the orphan granzymes to this process. Granzymes also have many nonapoptotic roles (Romero and Andrade, 2008; Trapani, 1998), and this aspect of immune function is still under investigation but may include a role in directly influencing viral replication (Andrade et al., 2007; Waterhouse and Trapani, 2007). Granzymes may also exhibit various polymorphisms. The specific reason for this and the function of the different polymorphic variants are currently under investigation.

Ultimately, a better understanding of the function of proteins involved in CL-mediated cell death is required so that we may effectively harness the immune system for better immune-based therapies for viral clearance, postviral tissue damage, and cancer.

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11

Cell Death in Nervous System Development and Neurological Disease

Juying Li and Junying Yuan

Naturally occurring neuronal cell death is common during the development of the nervous system. Depending on the neuronal population, as many as 20% to 80% of all neurons produced during embryogenesis die before the animal reaches adulthood. This regressive event functions to primarily adjust the magnitude of each neuronal population to the size or functional needs of its target field and has been recognized as a major player in establishing the definitive form of adult nervous system. Although immature neurons die physiologically in large numbers during development, mature neurons are among the most long-lived cell types in mammals. Under pathological conditions, mature neurons might activate cell death mechanisms that are known to occur in various acute and chronic neurodegenerative diseases. Pathological neuronal cell death, however, occurs through more diversified pathways than that during development. In this chapter, we review the evidence that supports the role of apoptosis during neuronal development, as well as the possible role of non-apoptotic cell death in the nervous system. We discuss the relevance of different cell death mechanisms in several neurodegenerative diseases and the potential neuroprotective targets for treatment of these diseases.

1. NATURALLY OCCURRING NEURONAL CELL DEATH DURING DEVELOPMENT WHEN NEURONS ARE ESTABLISHING THEIR TARGETING CONNECTIONS

1.1. Death by trophic factor deprivation

During nervous system development, the temporal phase of extensive neuronal cell death is usually confined to a well-defined period that is distinctive for each neuronal population and often coincides with the time

when the neuronal population as a whole is beginning to establish connections in its projection field. Many further experiments, which are summarized in Figure 11-1, supported the view that the target field is critical in determining the number of projecting neurons that survive. The survival of neurons during this period is highly dependent on the success of each neuron in competition for the availability of neurotrophic factors produced by their target or neighboring cells. The net result of such competition for trophic factor supply is the survival of some and death of other neurons, thereby matching the size of the target field with the number of innervating neurons. The programmed cell death of neurons also meets other adaptive needs, which include establishing optimal levels of connectivity between neuronal populations, eliminating aberrant cells or connections, regulating the size of progenitor populations, and serving transient functional needs of immature animals.

The first neurotrophic factor, nerve growth factor (NGF), was identified by Levi-Montalcini and Hamburger in 1951. Since then, the molecular mechanism of target-dependent neuronal cell death has been studied extensively in sympathetic neurons that are dependent on NGF for survival both in vivo and in vitro. In a frequently used paradigm, sympathetic neurons from superior cervical ganglion (SCG) of the embryonic day 21 rats are maintained in the presence of NGF for 5 to 7 days; the removal of NGF induces apoptotic cell death of all neurons 24 to 48 hours after NGF deprivation in culture. Inhibition of RNA and protein synthesis blocked apoptotic cell death, suggesting that neuronal apoptosis requires the participation of cellular protein synthesis activity and therefore might represent an active form of cell death.

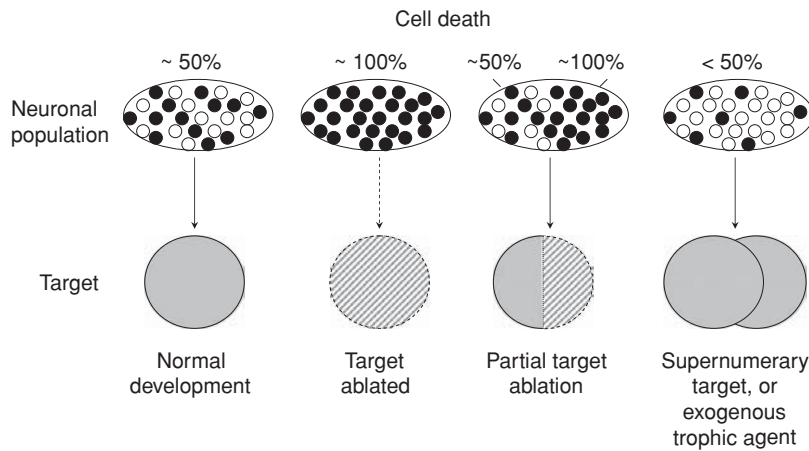


Figure 11-1. Major features of naturally occurring neuronal death during development. In most regions, approximately 50% of the neurons that are initially generated die at about the time when the population as a whole begins to form connections within its target field. If the target is partially or totally ablated, neurons are increasingly lost proportionally to the amount of target removed over the same time period. Expanding the target field or providing an exogenous trophic factor rescues some of the neurons that might be expected to die. Adapted from Cowan, W. M., J. W. Fawcett, et al. (1984). Regressive events in neurogenesis. *Science* 225(4668): 1258–65. Reprinted with permission from AAAS.

1.2. Key molecules regulating neuronal apoptosis during development

The breakthrough for understanding the mechanism of neuronal cell death induced by trophic factor deprivation came from the studies in the nematode *Caenorhabditis elegans*. During *C. elegans* development, of 1,090 somatic cells (of which 302 are neurons and 56 are glial cells), 131 undergo programmed cell death. The death of these 131 cells is predetermined by their genetic lineages and occurs at predictable times during the development of each individual worm. Thus programmed cell death in *C. elegans* is a form of cellular suicide. In contrast, there is no evidence to indicate that the death of mammalian neurons occurring during the period when they are establishing synaptic connection is predetermined. As discussed before, the verdict regarding which neuron will die and which will live is reached through a competition process during which neurons compete for establishing the correct synaptic connection and trophic factor availability. Therefore, mammalian neurons that die during the period of establishing synaptic connection are not predetermined by their lineage; rather, they are induced as a result of lacking trophic factor support.

The central components of the programmed cell death machinery in *C. elegans* are three CED proteins: CED-3, CED-4, and CED-9. In this cellular suicide machinery, CED-9 functions as an inhibitor of apoptosis by preventing CED-4 from interacting with CED-3, whereas CED-4 is a proapoptotic adaptor molecule

required for the activation of CED-3, a cysteine protease responsible for the execution of cell death program. In mammals, the homologs of CED-9, CED-4, and CED-3 are members of the Bcl-2 family, Apaf-1/NOD-like receptor family, and caspase family, respectively. The demonstration that neuronal cell death induced by the lack of trophic factors can be prevented by overexpression of Bcl-2 or by expression of a virally encoded caspase inhibitor crmA provided the first insights into the role of apoptosis in regulating neuronal cell death. Thus, surprisingly, although mammalian neuronal cell death induced by trophic factor deprivation occurs through a stochastic process of competition, they are regulated by a cellular suicide machinery that is very likely to share the same evolutionary origin as that of programmed cell

death in *C. elegans* that is predetermined by their cell lineages during development. Next we review the mechanisms by which neuronal cell death is regulated and executed.

1.2.1. Roles of caspases and Apaf-1 in neuronal cell death

Multiple virally encoded caspase inhibitors, such as crmA and p35, provided useful tools for demonstrating the roles of apoptosis in a variety of cellular systems, including neurons. The expression of crmA (a caspase inhibitor encoded by cowpox virus) in chicken dorsal root ganglion (DRG) neurons or p35 (a caspase inhibitor encoded by baculovirus) in rat sympathetic neurons inhibits apoptosis on NGF deprivation in culture. Peptide-based chemical inhibitors derived from the preferred caspase cleavage sites in their substrates also block neuronal cell death in vitro. Newborn DRG neurons from mutant caspase-1 (C285G) transgenic mice and caspase-1^{-/-} mice are resistant to trophic factor withdrawal-induced apoptosis in culture. However, it has been challenging to demonstrate the precise roles of caspases in regulating neuronal cell death during the period of establishing synaptic connection in vivo because until now, no caspase-deficient mice have shown a significant defect in the elimination of developing postmitotic neurons while establishing synaptic connections in vivo, despite the establishment of almost all caspase mutant mice.

Genetic analysis of caspase-deficient mice demonstrated the roles of caspase-3 and caspase-9 in mediating apoptosis of mitotically active neural progenitor cells or immature neurons in the forebrain during early developmental stages before the formation of synaptic connections. Caspase-3^{-/-} mice in mixed 129/SvJ and C57BL/6 background die perinatally with a variety of hyperplasia and disorganized cell deployment in the brain, similar to that of caspase-9^{-/-} mice. Ectopic cell masses appear in the cerebral cortex, hippocampus, and striatum, whereas the incidence of pyknosis, a prominent feature of apoptosis during normal neurogenesis in the periventricular zone, is significantly reduced in caspase-3^{-/-} and caspase-9^{-/-} mice. Certain mutant phenotypes of caspase-deficient mice, however, can have a strong dependency on genetic background (e.g., caspase-3^{-/-} mice are viable and developmentally normal in C57BL/6 background). The interaction of genetic background with a specific caspase deficiency might be a subject of interest for future studies.

The death of newborn neural precursor cells in the periventricular zone as those impaired in caspase-3^{-/-} and caspase-9^{-/-} mice, however, occurs before the time of establishing synaptic connection because neurons are born in the periventricular zone and the neuronal connections are only made after their migration to the appropriate layers of the brains. The regulation of neuronal cell death in population *in vivo* before the formation of synaptic connections may differ from the well-known target-dependent type of naturally occurring neuronal death that occurs when synaptic connections are being formed. Interestingly, in contrast to the striking perturbations in the morphology observed in the more rostral, mitotically active regions of the brain of caspase-3^{-/-} and caspase-9^{-/-} embryos, the spinal cord, brainstem, and peripheral ganglia in these caspase mutant mice appear completely normal at both embryonic and postnatal stages. Developing postmitotic neurons at these regions ultimately undergo normal number of neuronal loss, despite the temporal delay in cell death. Thus, although the studies using *in vitro* model of trophic factor-dependent neuronal cell death predict an important role of caspases in apoptosis, there is a general lack of evidence for the involvement of any specific caspase in postmitotic neuronal death *in vivo*. There might be a number of reasons to explain this. First of all, it is possible that the constitutive loss of a caspase in the germline might lead to upregulated expression of other caspases in the mutant background, which might compensate for the loss of a single caspase. This has been demonstrated for caspase-3^{-/-}, caspase-7^{-/-}, and caspase-9^{-/-} mice. However, a

compensatory expression of other caspases makes it difficult to explain the lack of obvious deficiency in the death of all neurons since the majority of late-stage caspase-3^{-/-}; caspase-7^{-/-} double knockout embryos, which lack both major downstream caspases required for the execution of apoptosis, did not show abnormality in brain morphology. It will be interesting to examine whether conditional caspase mutant mice that are specifically deficient for a caspase in neuronal lineage or in a temporally regulated manner show a defect in neuronal cell death induced by trophic factor deprivation. On the other hand, it is possible that in caspase-deficient mice, additional caspase-independent cell death mechanism is activated to compensate for the loss of caspase deficiency. In fact, although many populations of developing postmitotic neurons are able to exhibit normal amount of cell death in the absence of either caspase-3 or caspase-9, the morphology of these degenerating neurons differs from the more typical apoptotic cell death, and the kinetics of their degeneration is delayed. Ultrastructural analysis of degenerating spinal cord neurons from E14.5 caspase-3^{-/-} embryos revealed the presence of extensive cytoplasmic vacuoles that are not usually detected in apoptotic cells and that are seldom observed in dying neurons of control embryos. A delay in the degeneration of caspase-deficient neurons suggests that caspase-mediated neuronal cell death is more efficient than caspase-independent cell death.

Similar to caspase-9^{-/-} mice, regardless of genetic background, *apaf-1*^{-/-} mice also display a massive overgrowth of cells in the brain (exencephaly) that was attributed in part to the reduced cell death of both immature neurons and dividing neuronal precursor cells and that is consistent with the role of *apaf-1* being an activator of caspase-9. In mature neurons, however, *Apaf-1* was found to be dispensable for neuronal cell death caused by the lack of trophic signaling input from *TrkA* deficiency or synaptic activity from *Munc-18* deficiency *in vivo*. Many populations of postmitotic, "target-dependent" neurons, including spinal and cranial motor neurons, spinal interneurons, DRG sensory neurons, and sympathetic neurons in the SCG, undergo a quantitatively normal amount of cell death in the absence of *apaf-1*, although caspase-3 activation is blocked. The degenerating *apaf-1*^{-/-} neurons show numerous vacuoles atypical for apoptosis, suggesting the activation of a back-up cell death mechanism in developing neurons when caspase activation is inhibited. Thus it is possible that in postmitotic mature neurons that are deficient for caspase activation mechanism, the lack of trophic factor support might trigger an active form of cell death mediated through a caspase-independent mechanism.

1.2.2. Role of Bcl-2 family members in neuronal cell death

The Bcl-2 family includes both antiapoptotic and proapoptotic proteins that contain one or more Bcl-2 homology (BH) domains. Bcl-2 and Bcl-xl are two major antiapoptotic members of the Bcl-2 family. Over-expression of Bcl-2 prevents apoptosis of sympathetic neurons induced by NGF deprivation in culture. Transgenic mice expressing Bcl-2 in the nervous system show reduced neuronal cell death during developmental hypertrophy of the brain and increased numbers of facial motor neurons and retinal ganglion cells. It is interesting, however, to compare the neuronal phenotypes of the mice over-expressing Bcl-2 with that of caspase-9^{-/-} and apaf-1^{-/-} mice: although they all show reductions in neuronal cell death and increases in neuronal cell numbers, over-expression of Bcl-2 in the brains results in larger brains with more neurons but otherwise normal brain morphology, whereas caspase-9 deficiency or apaf-1 deficiency leads to severe defects in brain morphogenesis. Given the brain morphology of Bcl-2 transgenic mice, one might argue that a simple reduction in neuronal cell death is not sufficient to alter brain morphogenesis. By the same reasoning, it is possible that caspase-9 and apaf-1 have additional functions unrelated to regulation of apoptosis, a possibility that should be examined in the future.

The expression of Bcl-2 is high in the central nervous system during development and downregulated after birth; however, the expression of Bcl-2 is retained in neurons of the peripheral nervous system throughout life. Although the prenatal development of the nervous system in Bcl-2^{-/-} mice is normal, there is a subsequent loss of motor, sensory, and sympathetic neurons after birth, suggesting that Bcl-2 is crucial for the maintenance of specific populations of neurons during the early postnatal period.

The normal development of nervous system in Bcl-2^{-/-} mice might be attributed to the redundancy in the functions served by other members of the Bcl-2 family. Bcl-xl appears to be a good candidate because it is also expressed in the developing brain. Unlike Bcl-2, whose expression decreases after birth, Bcl-xl expression is retained in the neurons of the adult central nervous system. Bcl-xl^{-/-} mice die around embryonic day 13, with extensive apoptotic cell death in postmitotic differentiating neurons of the developing brain, spinal cord, and dorsal root ganglia. Striking deficiency of neuronal survival in Bcl-xl^{-/-} mice indicates its pivotal role in maintaining neuronal survival.

Mcl-1 is another important antiapoptotic Bcl-2 family member. Mcl-1^{-/-} mice die very early in embryonic development around embryonic day 3.5, which is the most severe phenotype among all the known mutant mice in the members of antiapoptotic Bcl-2 family. Robust expression of Mcl-1 is present in both proliferating neuronal progenitor cells and postmitotic neurons during brain development. Mcl-1 deficiency, especially in neuronal lineage, results in the apoptotic death of both Nestin⁺ neural progenitors and Tuj1⁺ newly committed neurons. During the development of the nervous system, proapoptotic Bax and bid are expressed in neural precursor cells within the ventricular zone, with the expression of Bax peaking at E12 to E15, corresponding to the period of early neurogenesis when widespread apoptosis of neural precursors occurs in the Mcl-1 conditional mutants. Although both Mcl-1 and Bcl-xl have been shown to block Bax- and Bak-mediated apoptosis, Bcl-xl is expressed at very low levels in the neural precursor populations, and apoptosis is observed in more mature neuronal populations in the Bcl-xl^{-/-} mutant mice at E12.5. Therefore, Mcl-1 plays an important role in regulating the survival of neurons during the transition from the progenitor to the postmitotic period.

Antiapoptotic members of the Bcl-2 family act as antagonists for the proapoptotic members of Bcl-2 family. Bax is a key member of proapoptotic Bcl-2 family in regulating neuronal apoptosis. Wild-type neonatal sympathetic superior cervical ganglion neurons and facial motor neurons express Bax mRNA at a time when these neuronal populations are susceptible to growth factor deprivation in vivo. Deletion of Bax results in profound effects on the survival of many kinds of neurons. Bax^{-/-} neonatal sympathetic neurons and facial motor neurons survive nerve growth factor deprivation in culture and disconnection from their targets by axotomy in vivo, respectively. Bax^{-/-} mice have increased neuronal numbers in the superior cervical ganglia and facial nuclei. Thus the activation of Bax may be a critical event for neuronal cell death induced by trophic factor withdrawal, as well as injury. Further studies examined the fate of the excess rescued neurons in postnatal Bax^{-/-} mice during muscle target innervation and revealed that although initially all of the motor neurons, including those rescued by Bax deletion, are able to project to and innervate targets, only a subpopulation can grow and retain target contacts postnatally. Treatment with exogenous trophic factor can reverse their atrophy and promote regrowth of the axons of the excess surviving motor neurons, suggesting that even after their developmental role in

regulating motor neuron survival is completed, trophic signals continue to be required to promote cell growth and to sustain target innervation.

1.3. Signal transduction from neurotrophins and neurotrophin receptors

The interactions of members of neurotrophin family, including NGF, brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3, NT-4/5, and NT-6/7 with their receptors, TrkA, TrkB, TrkC, and p75NTR (p75 neurotrophin receptor), play important roles in the development of the nervous system and the maintenance and repair of adult nervous system. The outcome of such neurotrophin-mediated interactions is dependent on the cell type and the expression pattern of each neurotrophin receptor. The activation of tropomyosin receptor kinase (Trk) receptors induces survival and differentiation of neurons. The activation of p75NTR in the absence of a strong Trk signal induces apoptosis of neurons, whereas in the presence of Trk signaling, p75NTR acts a coreceptor to enhance response to neurotrophins. Although the role of targets in controlling neuronal cell death historically has received the most attention, signals derived from afferent inputs and nonneuronal cells such as central and peripheral glia and endocrine glands are also possible sources of trophic regulation of cell death and survival.

1.3.1. Signals for survival

Trk receptors belong to the receptor tyrosine kinase family that regulates synaptic strength and plasticity in the mammalian nervous system. The three most common types of Trk receptors are TrkA, TrkB, and TrkC. Each of these receptors types has different binding affinity to certain types of neurotrophins, which are trophic factors mediating neuronal survival. Each neurotrophin family member is synthesized as an approximately 250–amino acid precursor (proneurotrophin) that is processed into a roughly 120–amino acid mature neurotrophins by furins and prohormone convertases. Secreted neurotrophins form homodimer and activate Trk receptors by promoting receptor dimerization. On binding of NGF, Trk receptors autophosphorylate in the cytoplasmic tail, which forms the docking sites for downstream signaling molecules such as phospholipase C γ , phosphoinositide 3-kinase (PI3K), and adaptor proteins such as Shc (Figure 11-2). The activation of PI3K and mitogen-activated protein (MAP) kinases play important roles in regulating neuronal survival.

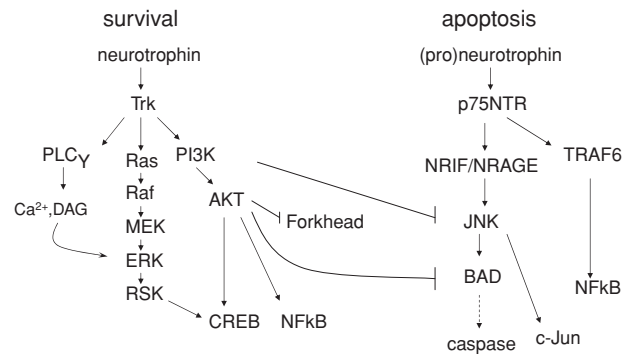


Figure 11-2. Trk and p75NTR signaling pathways. Neurotrophins bind Trk receptors or p75NTR to activate survival or apoptotic signaling pathways. On ligand binding, activated Trk receptors dimerize and autophosphorylate the cytoplasmic domains, which provides docking sites for three principal downstream signaling pathways, phospholipase C pathway, Ras-MAP kinase pathway, and PI3K-Akt pathway. These pathways lead to nuclear translocation of transcription factors, such as CREB and NF κ B, and ultimately regulation of gene expression. Phosphorylation of the members of Forkhead family inhibits their translocation into the nuclei, which reduces the expression of Forkhead target genes that promote apoptosis. The binding of neurotrophins or proneurotrophins to p75NTR can activate Bad via JNK cascade and eventually leads to the release of effectors from mitochondria and caspase activation. There are cross-talks between the survival and proapoptotic signaling cascades.

The role of the PI3K pathway in neuronal survival was initially suggested by the observation that PI3K inhibitors block the ability of NGF to prevent apoptosis in a neuron-like cell line, PC12 cells. Activation of PI3K leads to the production of the various 3-phosphorylated phosphoinositide lipid signaling molecules, among which either phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P3) or phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P2) binds and regulates the serine/threonine protein kinase Akt. Later studies on SCG neurons showed that either PI3K activation or constitutively active Akt expression keeps sympathetic neurons alive in the absence of NGF. Activated Akt mediates the phosphorylation of proapoptotic protein Bad and caspase-9, which contributes to the inhibition of apoptosis. Activated Akt also promotes cell survival by regulating at least three transcription factor families: Forkhead, cAMP-response element-binding protein (CREB), and nuclear factor kappa B (NF- κ B). Phosphorylation of the members of Forkhead family inhibits their translocation into the nuclei, which reduces the expression of Forkhead target genes that promote apoptosis, such as the Fas ligand. Phosphorylation of CREB and I κ B kinase (IKK) stimulates the transcription of prosurvival genes by CREB and NF- κ B.

Activated Trk receptors provide the docking site for Shc, which triggers the activation of the Ras-Raf-MAP

kinase signaling cascade. The effect of the MAP kinase pathway on cell survival is mediated at least in part by the activation of downstream pp90 ribosomal S6 kinase (RSK) family members. Like Akt, RSK has been shown to phosphorylate and inhibit Bad. RSKs are also potent activators of CREB, which activates the transcription of the antiapoptotic gene *Bcl-2* and a variety of immediate-early genes and delayed-response genes in regulating cell survival, axonal and dendritic growth, and neuronal differentiation. Thus PI3K-Akt and MAP kinase pathways converge on Bad and CREB to inhibit the apoptosis program.

1.3.2. Signals for death

The removal of NGF leads to a rapid inhibition of PI3K and MAP kinase activities, followed by a series of early metabolic changes, including increased production of reactive oxygen species, decreased glucose uptake and decreased RNA and protein synthesis, and increased c-Jun N-terminal kinase (JNK) activation or c-Jun phosphorylation.

The physiologic role of NGF in many systems is to promote neuronal survival by acting through the high-affinity TrkA receptor tyrosine kinase. NGF also acts through low-affinity receptor p75NTR during development to negatively regulate numbers and cholinergic phenotype of forebrain cholinergic neurons, because the number and the size of these neurons are increased in p75NTR^{-/-} mice or in normal mice treated with a peptide that inhibits the binding of NGF to p75NTR. p75NTR is a member of the tumor necrosis factor (TNF) receptor superfamily. It binds mature neurotrophins with lower affinity, but binds pro-neurotrophins with higher affinity compared with that of Trk receptors. The neurotrophin-bound p75NTR can associate with several signaling partners, including Nogo receptor, sotrillin, and LINGO-1, which are involved in regulating neurite outgrowth in response to myelin proteins such as Nogo, MAG, and OMgp. Formation of these different platforms may explain the multiple effects of p75NTR in different cell types and contexts.

The intracellular domain of p75NTR contains a region that bears similarity with the death domain of the TNF receptor family. In the condition of low or no Trk activity, highly activated p75NTR signals through ceramide, the JNK family, and NF- κ B, similar to other members of TNF receptor family, to induce apoptosis. However, in the presence of high Trk activity, p75NTR-mediated apoptosis is suppressed. Instead, coactivation of p75NTR enhances Trk-mediated cell survival and differentiation. Concurrent stimulation by neurotrophins of p75NTR

and Trk receptors constitutes a dual growth control with antagonistic and synergistic elements aiming at optimal functional integration of cells and cell populations into their context.

2. PATHOLOGIC NEURONAL CELL DEATH IN THE ADULT BRAIN

In the first part of this chapter, we discussed programmed neural cell death during development, which is required to achieve the accurate wiring of the nervous system. However, genetic or environmental factors can lead to nonprogrammed death of neurons during adult life due to neural insults caused by the onset of neurodegenerative disorders, stroke, or trauma. Pathological neuronal death can occur by apoptosis, necrosis, or a combination of both. The manner by which neuronal cell death is executed in a particular condition may depend on several factors, including the neuronal cell type involved, the nature and severity of the insult, and the energy content of the cells. For example, it has been shown that neurons at the core of an ischemic lesion undergo necrotic death and cannot be rescued by treatment with caspase inhibitors, whereas apoptotic neurons are more likely to be found at the penumbra, and cell death can be prevented in the presence of caspase inhibitors. In this part, we first discuss the evidence of apoptotic death in several neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS). We also provide evidence for the involvement of caspase-independent cell death in neurodegenerative disorders, focusing on the proteolytic mechanism of calpains and cathepsins.

2.1. APOPTOSIS IN NEURODEGENERATIVE DISEASES

2.1.1. Alzheimer's disease

AD is the most common form of dementia. A central role for amyloid- β (A β) protein in AD progression is supported by the effects of genetic mutations responsible for familial AD, which predisposes to amyloid plaque deposition in the brain. A β , a peptide that forms amyloid plaques, can directly induce apoptosis of cultured neurons. Fragmented nuclear DNA has been detected in brains of AD patients by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Biochemical evidence that supports the involvement of apoptosis in AD is provided by the detection of activated caspase-3, -8, and -9 in the hippocampal neurons of the brains affected by AD. Moreover, pharmacological

inhibition or genetic mutations of caspase family members, such as caspase-2, -3, -8, and -12, have been reported to offer partial or complete protection against A β -induced apoptotic cell death *in vitro*.

A β is a proteolytic cleavage product of γ -secretase presenilin-mediated processing of amyloid precursor protein (APP). Mutations in presenilin genes, responsible for a significant subset of early-onset familial AD, increase the production of a 42-residue form of A β , which is a major constituent of plaques in the AD patient brain, and neuronal sensitivity to apoptosis. In both cell culture and AD-affected brains, APP can also be cleaved by caspases, such as caspase-3, at the sites distinct from the presenilin-cleavage sites. Caspase-mediated cleavage of APP not only releases A β , but also releases a carboxy-terminal peptide that is a potent inducer of apoptosis. Caspase-3 cleaved fragments of tau, a microtubule-associated protein that is the primary protein component of the filaments found in AD patient brains, have also been detected in postmortem samples. Despite all the evidence that supports a role of apoptosis in AD, questions have been raised regarding how apoptosis, a rapid form of cell death, might be compatible with the chronic progression of AD. In the AD brain, some neurons exhibit morphological features of apoptosis, but many degenerating neurons do not show evidence of apoptosis, suggesting that apoptosis might not be the only mechanism of degeneration in AD. Furthermore, evidence obtained from postmortem brain samples should be viewed with caution because increased caspase activation might have occurred in the context of a diseased brain postmortem.

Although the mechanism of A β neurotoxicity and its precise cellular locus of action are not settled, the evidence supporting the involvement of A β in AD is strong. A β has also been shown to induce oxidative stress and elevate intracellular Ca²⁺ levels, which activates several cell death signaling pathways. In addition, the increased presence of activated microglia, a prominent feature of AD patient brains, indicates the activation of inflammatory response. Microglial activation is associated with amyloid plaques and can be induced experimentally by A β . Microglial activation induced by A β leads to the secretion of TNF- α and other toxic factors that can induce neuronal apoptosis. Thus pathological neuronal death in AD might be a consequence of a complex interaction between neurons, microglia, and toxic factors.

2.1.2. Parkinson's disease

PD is characterized by resting tremor, slowness of movement, rigidity, and postural instability. These symptoms

are attributed to the loss of dopamine (DA)-containing neurons in the substantia nigra pars compacta (SNPC). Biochemical assessment of apoptotic markers in PD patient brains revealed that both proapoptotic proteins, e.g. Bax, caspase-3, caspase-8, caspase-9, and antiapoptotic proteins (e.g., Bcl-xl) show increased expression or activation in DA neurons as compared with that of controls.

Early research using a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced mouse model of PD has provided evidence for the possible involvement of apoptosis in the pathogenesis of PD. MPTP, a byproduct during the chemical synthesis of a meperidine analogue, has potent heroin-like effects that can induce a disease syndrome in humans almost indistinguishable from PD. A metabolic product of MPTP, MPP⁺, is concentrated in the mitochondria of DA neurons, where it inhibits the complex I of electron transport chain and results in an increased production of reactive oxygen species. Prolonged administration of a low dose of MPTP to mice induces downregulation of Bcl-2, upregulation of Bax, activation of caspase-9 and caspase-3, and morphologically defined apoptosis in DA neurons. Bax^{-/-} mice and Bcl-2 transgenic mice are resistant to the toxicity of MPTP. The activation of p53 plays an important role in mediating the death of DA neurons after MPTP intoxication, as p53^{-/-} mice are resistant to the MPTP-induced DA neuron death. In addition, pharmacological blockade of JNK activation results in a marked attenuation of MPTP-induced neurodegeneration. Blockage of the intrinsic apoptosis pathway (mitochondria pathway) by an intrastriatal injection of an adeno-associated viral vector containing a dominant-negative form of Apaf-1 also prevents the MPTP-induced activation of caspase-3 and SNPC neuronal death.

α -synuclein is an important component of the intracellular inclusions known as Lewy bodies, which are the neuropathological hallmark of PD. Dominantly inherited gain-of-function mutations in α -synuclein have been found in a subset of familial PD. Although the mechanism by which mutations in α -synuclein induce DA cell death has not been well established, deletion of α -synuclein in mice prevents MPTP-induced neurodegeneration, whereas α -synuclein transgenic mice show increased sensitivity to the toxin, and expression of mutant α -synuclein in cell cultures promotes apoptosis.

Recent progress in molecular genetics has identified several genes implicated in familial forms of PD, including α -synuclein, leucine-rich repeat kinase 2 (LRRK2), Parkin, DJ-1, and phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1), many of them coding for proteins found in Lewy bodies and/or implicated in

mitochondrial function. Animal models based on genetically modified mice with null mutation, an extra gene copy, or point mutations of these genes have helped us to obtain insights into the mechanism of several symptoms of PD. However, none of the genetic models based on PD-linked genes recapitulate the key symptom of the disease, such as the loss of dopamine neurons, but more subtle effects on the DA system have been detected. α -synuclein transgenic mice and DJ-1 knockout mice were reported to be more susceptible to MPTP toxin, suggesting that the progression of PD might be due to a combination of genetic factors and environmental exposures.

It is noteworthy that targeting apoptosis upstream of its execution phase results in a marked attenuation of neurodegeneration in PD animal models, whereas interfering at a more downstream level, such as caspase activation, produces variable results. Inhibition of caspase activation by gene transfer of X-linked inhibitor of apoptosis or peptide inhibitors prevents the loss of DA neuron cell body but not nerve fibers. Because the symptoms of PD are caused by the loss of DA terminals in the striatum, preventing the death of DA cell bodies without preventing the degeneration of their axons is unlikely to be a good therapeutic strategy. A combinatorial strategy might be required to prevent both the loss of cell body and axon degeneration to obtain clinical benefits.

2.1.3. Huntington's disease

HD is an autosomal-dominant neurodegenerative disease characterized by involuntary movements and dementia that result from selective neuronal loss in the striatum and cerebral cortex. HD is caused by expansions of CAG in the *huntingtin* gene, producing a protein containing elongated poly-glutamine (poly-Q) repeats. The length of poly-Q repeats shows a rough inverse correlation with the ages of disease onset, suggesting the length of poly-Q is an important factor in the pathogenesis of this disease. Proteins with poly-Q repeats can aggregate in vitro and in vivo. Transgenic mice that over-express an N-terminal fragment of human huntingtin with expanded poly-Q region show reduced survival, intraneuronal aggregates, and behavior deficits similar to HD. The oligomerization of expanded poly-Q repeats play a pivotal role in the neurodegeneration of HD.

Caspase activation has been proposed to participate in the mutant huntingtin-mediated cell death. TUNEL-positive cells and caspase-1 and caspase-8 activation have been detected in the HD patient brains. In mouse models of HD, expression of a dominant-negative form of caspas-1 or the intracerebroventricular

administration of a pan-caspase inhibitor zVAD-fmk delays the progression and mortality of the disease.

How mutant huntingtin triggers apoptosis remains unclear. Expression of expanded poly-Q repeats in vitro can directly activate caspase-3, -8, and -9. Caspase-3 and caspase-6 can also cleave wild-type and mutant huntingtin proteins, generating truncated fragments. Truncated fragments that contain expanded poly-Q repeats show increased toxicity and propensity to aggregate compared with full-length protein. Caspase-8 has been found to be recruited into the intracellular aggregates and is activated in neuronal cells that express expanded poly-Q repeats. Caspase-8 activation in HD is mediated by the formation of heterodimers between Hip1 (huntingtin-interacting protein 1) and Hippi (Hip1 protein interactor), which is favored by the disease-associated poly-Q expansion. Despite the insights offered by these studies, we need to be cautious when considering caspases as therapeutic targets for HD because the physiologic relevance of caspase-mediated cleavage of huntingtin in vivo remains to be established.

A central issue is the relative contribution of neuronal apoptosis to neurological deficits in HD and other age-related neurodegenerative disorders. Early-stage HD patients develop characteristic motor deficits without evidence of striatal atrophy; striatal atrophy becomes prominent only in later stages of the disease. Furthermore, in a conditional huntingtin transgenic mouse, neurological deficits could be reversed by turning off expression of the mutant transgene. Thus neural dysfunction, rather than irreversible cell death, might be responsible for early neurological deficits. This conclusion suggests that the primary target for the therapy of HD should be the mechanism of neural dysfunction, rather than that of cell death.

2.1.4. Amyotrophic lateral sclerosis

ALS is a fatal disorder characterized by the loss of motor neurons in the cerebral cortex and spinal cord, leading to progressive and ultimately fatal paralysis. A major advance in the understanding of the disease mechanism came from the identification of mutations in the gene encoding superoxide dismutase (SOD1) that is responsible for a significant portion of familial ALS cases. Mice deficient in SOD1 do not develop any motor neuron disease. Transgenic expression of different human ALS-linked *SOD1* mutations in mice and rats replicates the clinical and pathological characteristics of ALS, without a correlation on the free radical scavenging activity of SOD1. These indicate that the cytotoxicity of mutant SOD1 is a gain of function. It has been

shown that mutant SOD1 can form intraneuronal aggregates and induce oxidative stress. The mechanism by which mutant SOD1 induces ALS appears to be complex because it may involve cell-autonomous effects in motor neurons that may determine the onset of the disease, as well as effects in nonmotor neurons, such as microglia, astrocytes, and oligodendrocytes, which affect the disease progression.

A role for apoptosis in ALS is suggested by the proapoptotic activity of mutant SOD1 in cultured neuronal cell lines and the neuroprotective effect of overexpressing Bcl-2 in mutant SOD1 transgenic mice. In the lumbar cord of patients with ALS and mutant *SOD1* transgenic mice, the mRNA content and protein level of Bcl-2 are decreased, whereas those of Bax mRNA are increased as compared with that of control. Activated caspase-1 and caspase-3 can be detected in the spinal cords of ALS patients and mutant *SOD1* transgenic mice. Inhibition of caspase-1 activity delays disease progression and prolongs the life span in *SOD1* transgenic mice. Evidence for a prominent recruitment of the mitochondrial pathway has been found in the spinal cord of patients and transgenic *SOD1* mice, and the treatment with minocycline, which has an inhibitory effect on mitochondrial dysfunction, delays disease onset and extends survival of the transgenic *SOD1* mice. Bcl-2 and mutant SOD1 protein physically interact in spinal cord mitochondria. Motor neurons isolated from mutant SOD1 transgenic mice show increased susceptibility to the activation of the Fas-triggered cell death pathway, suggesting the extrinsic pathway might also make a contribution to the neurodegenerative process.

Although the studies just discussed support the involvement of apoptosis in the death of motor neurons involved in ALS, they are by no means conclusive. As with other chronic neurodegenerative diseases, neural dysfunction, which occurs considerably earlier than that of neuronal cell death, may be responsible for the onset of ALS, whereas neuronal cell death is only manifested in late stage of the disease. Although Bax deletion prolongs survival and completely blocks mutant SOD1-mediated motor neuron cell death, it only delays the timing of neuromuscular denervation, which began long before the activation of cell death proteins in SOD mutants. Expression of wild-type or mutant human SOD1 in the motor neurons of *Drosophila* induced progressive climbing deficits associated with defective neural electrophysiology, focal accumulation of SOD1, and a stress response in surrounding glia without a loss of neurons. Thus maintaining normal neural connectivity and function should be an important goal for the treatment of chronic neurodegeneration such as ALS.

2.2. Necrotic cell death in neurodegenerative diseases

Necrosis typically occurs after acute neurological injuries, such as ischemia, hypoxia, stroke, or trauma, as well as in chronic neurodegenerative diseases, including AD, PD, HD, and ALS. Although the molecular mechanisms of necrotic cell death are mostly not understood, elevated intracellular calcium, cytosolic calpains, and spilled lysosomal cathepsins are the major players in necrotic neuronal death.

2.2.1. Calpains

Calpains are a family of calcium-dependent cysteine proteases that cleave a variety of cellular substrates. The cross-talk between caspases and calpains has been reported in a numbers of in vivo and cell culture models of apoptosis. Calpain-mediated cleavage of caspases results in both caspase inhibition and activation. Conversely, caspases regulate calpain activity by mediating degradation of calpastatin, the endogenous inhibitor of calpain.

The importance of calpain activation in acute cell injury and necrotic cell death triggered by calcium influx has been established. One of the mechanisms by which calpain activation contributes to cell death is the cleavage of several cytoskeletal proteins of neuronal axons, such as neurofilaments, cain/cabin 1, and fodrin. Degradation of neurofilaments induced by oxygen/glucose deprivation could be attenuated by calcium removal, blockade of voltage-gated sodium channels, or inhibition of calpains. Another mechanism by which calpain contributes to cell demise is the cleavage of membrane channels (e.g., the subunit NR2B of the N-methyl-D-aspartate receptor and the plasma membrane Na/Ca exchanger [NCX]), during excitotoxicity. NCX operates in cellular calcium extrusion, and its proteolytic inactivation by calpain is responsible for the secondary phase of excitotoxic calcium upregulation and the death of the neurons.

The role of calpains in neuronal cell death has also been examined in chronic neurodegenerative diseases. Inhibition of calpains prevents neuronal and behavioral deficits in a mouse model of PD, and calpain activation was evident in postmortem midbrain tissues from PD patients. In the case of AD, calpain activation occurs before abnormalities in the microtubule-associated protein tau. Activated calpain associates with neurofibrillary tangles, which are abnormal aggregates of hyperphosphorylated tau and a major pathological feature of AD. Calpain activation has been detected in human HD caudate but not in age-matched controls.

Huntingtin protein is degraded to small fragments by calpain after ischemic injury. The huntingtin fragments generated from calpain cleavage are smaller than those from caspase cleavage and are more toxic.

2.2.2. Cathepsins

Cathepsins, which are two classes of lysosomal proteases including aspartyl (cathepsin D) and cysteine (cathepsin B, H and L) proteases, play key roles in neurodegeneration. Cathepsins have been implicated in both intracellular proteolysis and extracellular matrix remodeling. Dysregulation or absence of cathepsins has important consequences on the maintenance and function of the nervous system. Mice deficient in cathepsin B and L die 2 to 4 weeks after birth and display neuronal loss and brain atrophy. Cathepsin D deficiency induces a lysosomal storage disease in mouse central nervous system neurons and degeneration of neurons in the mouse retina.

In models of neurological disorders, cathepsins B and L have been implicated in delayed neuronal death after global and focal cerebral ischemia. Specific inhibitors of cathepsins B and L effectively reduce ischemia cerebral damage. Cathepsin B release is an early event after occlusion of cerebral arteries, which eventually triggers the activation of proinflammatory caspases, caspase-1 and caspase-11, in focal cerebral ischemia. Cathepsin D is involved in neuronal death induced by aging, transient forebrain ischemia, and excessive stimulation of glutamate receptors during excitotoxicity. Lysosome numbers and the concentration of cathepsin D increase in neurons that are vulnerable to AD before the onset of pathology.

What signals trigger the activation of multiple proteases that lead to demise of neurons in both acute and chronic neurodegenerative diseases? Because an increase in intracellular calcium and abnormal activation of calcium-regulated processes are among the most ubiquitous features in neurodegeneration, calpain activation represents a critical step in both apoptosis and necrosis. Mild calcium elevation favors apoptosis, whereas acute calpain activation precipitates necrosis, probably via catastrophic cleavage of regulatory and structure proteins. Studies in primates also show that calpains localize to lysosomal membranes after the onset of ischemic episodes, with subsequent spillage of cathepsins to the cytoplasm to execute necrosis. Protease activation in neurodegeneration is very complex and is likely to involve cross-talks of caspases, calpains, and cathepsins in a manner depending on the neuronal population and the nature or severity of the insult.

3. CONCLUSIONS

In this chapter, we discussed the roles of the key components of the apoptosis program in neuronal development, Apaf1, Bcl-2 family proteins, and caspases. The role and mechanism by which neurotrophins suppress apoptosis and regulate cell survival signaling are well appreciated. Evidence has been presented that supports the involvement of activation of apoptosis in chronic neurodegenerative diseases such as AD, PD, HD, and ALS. However, it is important to understand that such evidence is by no means conclusive; other cell death mechanisms such as necrosis and other deleterious mechanisms may also contribute to the degenerative process. Furthermore, one must also understand that although neuronal cell death plays a very important role in the progression and in the final stages of these universally lethal diseases, it might not be the primary or sole factor that determines the onset of these chronic neurodegenerative diseases. In many neurodegenerative diseases, axonal dysfunction long precedes neuronal death. The molecular mechanisms underlying axonal damage are distinct from those underlying cell death. Additionally, an early loss of synapses has been observed in various animal models of developmental neurodegeneration. Therefore, targeting cell death alone is unlikely to be sufficient to obtain a beneficial therapeutic effect. Optimal neuroprotection might require administration of a pharmacological cocktail against multiple pathogenic events, including axonal/synaptic dysfunction, inflammation, and cell death.

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12

Role of Programmed Cell Death in Neurodegenerative Disease

Dale E. Bredesen

Death? Why this fuss about death? Use your imagination, try to visualize a world without death! ... Death is the essential condition of life, not an evil.

– Charlotte Perkins Gilman

1. INTRODUCTION: PROGRAMMED CELL DEATH, CELL DEATH SIGNALING, AND NEURODEGENERATIVE DISEASE

Many of the diseases that affect the nervous system feature an abnormality of cell death of one sort or another. For example, developmental and neoplastic disorders of the nervous system feature dysregulation of the intrinsic cellular programs that mediate cell death. Furthermore, there is increasing evidence to suggest that such dysregulation may also occur in neurodegenerative, infectious, traumatic, ischemic, metabolic, and demyelinating disorders. Therefore, targeting the central biochemical controls of cell survival and death may represent a productive therapeutic approach, especially if combined with other therapeutic strategies. Furthermore, recent results from stem cell studies suggest that the fate of neural stem cells may also play an important role in disease outcomes, and therefore, cell death apparently plays a central role in many neurological diseases and potentially in their prevention and treatment.

Early studies of neuronal survival focused on the status of external factors such as glucose availability, pH, and the partial pressure of oxygen. However, although these are clearly critical determinants, research over the past few decades has revealed a more active, and more plastic, role for the cell in its own decision to survive or die than was previously appreciated. Complementing this concept, studies of the internal suicide programs of neural cells have offered new potential targets for therapeutic development.

In neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, frontotemporal dementia, Huntington's disease, and amyotrophic lateral sclerosis (Lou Gehrig's disease), neurons in various brain or spinal cord nuclei are lost in disease-specific distributions. However, the neuronal loss is a relatively late

event, typically following synaptic dysfunction, synaptic loss, neurite retraction, and the appearance of other abnormalities such as axonal transport defects. This progression argues that cell death programs may play at best only a secondary role in the neurodegenerative process. However, emerging evidence from numerous laboratories has suggested an alternative possibility: that although cell death itself occurs late in the degenerative process, the pathways involved in cell death signaling do indeed play critical roles in neurodegeneration, both in sub-apoptotic events such as synapse loss and in the ultimate neuronal loss itself.

Although initial comparisons of the intrinsic suicide program in genetically tractable organisms such as the nematode *Caenorhabditis elegans* failed to disclose obvious relationships to genes associated with human neurodegenerative diseases (e.g., presenilin-1 does not bear an obvious relationship to the major cell death genes *ced-3*, *ced-4*, or *ced-9* in *C. elegans*), more recent studies support such a relationship. For example, the mammalian homologs of *ced-3* comprise a family of cell death proteases, the caspases, and mutation of a single caspase cleavage site in huntingtin blocks the development of the Huntington's phenotype in transgenic mice. A detailed understanding of the interrelationship between fundamental cell death programs and neurodegenerative processes is still evolving, and it promises to offer novel approaches to the treatment of these diseases.

2. MECHANISTIC TAXONOMY OF CELL DEATH: HOW MANY TYPES OF PROGRAMMED CELL DEATH CAN BE DISTINGUISHED?

Classical developmental studies support the view that at least three different programmed cell death (PCD) forms are distinguishable: type I, also called nuclear

or apoptotic; type II, also called autophagic; and type III, also called cytoplasmic. These occur reproducibly within specific neuroanatomical nuclei and with specific frequencies, at specific times of nervous system development. However, these developmental or physiologic cell death pathways may also be activated by various insults, such as ischemia, DNA damage, or the accumulation of misfolded proteins. Mechanistic requirements for type I cell death center on caspase-dependent pathways (extrinsic and intrinsic), though some have argued that cellular morphologies resembling apoptosis can occur independent of these proteases. Types II and III do not require caspase activation, but the possibility that they may in some cases be accompanied by caspase activation has not been excluded.

Beyond the three types of developmental cell death, other forms have been described that do not fit the criteria for any of them. For example, a nonapoptotic, caspase-independent form of cell death that does not resemble type II or type III developmental PCD has been described by Driscoll and colleagues in *C. elegans* that express mutant channel proteins, such as *mec-4(d)*, that mediate neurodegeneration. A uniform, necrosis-like cell death ensues, characterized morphologically by membranous whorls lacking in other cell death types, triggered by calcium entry, mediated by specific calpains and cathepsins, and inhibited by calreticulin. Although it is possible that this alternative form of PCD will ultimately turn out to proceed via one of the previously described pathways (e.g., type II or type III), the morphological characteristics suggest that it may be a distinct form of nonapoptotic PCD.

A fifth apparent form of PCD has been described by Yu, the Dawsons, and their colleagues, who showed that a nonapoptotic form of cell death depends on the activation of poly-(ADP-ribose) polymerase (PARP) and the consequent translocation of apoptosis-inducing factor (AIF) from mitochondria to nucleus. AIF is a flavoprotein, discovered by Kroemer and his colleagues, that is involved with DNA fragmentation, along with endonuclease G and DNA fragmentation factor. This form of PCD was shown to be activated by agents that induce DNA damage, such as hydrogen peroxide, N-methyl-D-aspartate, and N-methyl-N'-nitro-N-nitrosoguanidine. Just as in the case of the calcium-activated PCD referred to previously, PARP-dependent PCD displays a morphology and biochemistry that appear to be distinct from types I, II, and III PCD.

As additional data are gathered from other cell death paradigms, novel biochemical pathways of PCD may be characterized. For example, an extensive literature on the morphological criteria for another potential form of

PCD – oncosis – exists, but the biochemical mediators of oncosis have not yet been described. Oncosis refers to a specific morphology of cell death – cellular swelling – typically induced by ischemia and thought to be mediated by the failure of plasma membrane ionic pumps. One potential mediator of oncosis is a calpain-family protease (possibly a mitochondrial calpain). This finding suggests that oncosis may prove to be similar to the calcium-activated necrosis-like cell death described by Driscoll et al. Another potential PCD pathway has been referred to as *autoschizis*, a form of cell death shown to be activated in certain tumor cells after treatment with ascorbate and menadione. Altogether, these observations illustrate the difficulty of relying on cell morphology to define cell death mechanisms and suggest a wide diversity of possibilities.

3. PROGRAMMED CELL DEATH SIGNALING IN NEURODEGENERATION

Evidence for caspase activation in neurodegeneration has been derived both from the use of antibodies directed against newly exposed proteolysis-dependent epitopes (neo-epitopes) generated by caspase cleavage and from the inhibition of neurodegeneration by caspase inhibitors. However, some neurodegenerative models and diseases clearly demonstrate nonapoptotic forms of PCD as well. Determining which PCD pathways are triggered in each neurodegenerative disease, which pathway accounts for each fraction of cell death, the mechanism(s) by which each pathway is triggered, and the interactions of the various pathways should shed new light on the degenerative process and its potential treatment or prevention.

One of the critical goals for dissecting the relationship between PCD and neurodegeneration is to determine the specificity of the trigger: in other words, is PCD activated in neurodegeneration as the result of a relatively nonspecific toxic effect of a peptide or protein aggregate? If so, then secondary neurodegeneration may occur due to loss of trophic support, excitotoxicity, or any number of other secondary effects. Alternatively, by analogy to neoplasia, are specific, physiologically relevant transduction events that underlie neurite retraction and synapse loss triggered directly by neurodegeneration-associated transcriptional and post-transcriptional events? In other words, if neoplasia is the result of an imbalance in physiological signaling events involving oncogenes and tumor suppressor genes, is neurodegeneration an analogous process that is the manifestation of an imbalance in physiologic signals that mediate synaptic maintenance and synaptic

re-organization? Evidence on both sides exists: for example, numerous toxic properties have been attributed to the A β peptide implicated in Alzheimer's disease, such as reactive oxygen species generation and metal binding, among others. However, signal transduction effects have also been attributed to A β peptide, such as binding and multimerization of amyloid precursor protein, with resultant complex formation and direct caspase activation.

Because the neurodegenerative process may be induced by widely varying insults – from misfolded proteins to reactive oxygen species to caspase recruitment complexes, as well as other mechanisms – and yet produce a relatively small number of syndromes, the existence of a death network is suggested. Such a network may be entered from many different sites, but once triggered, would follow similar interdependent biochemical pathways, with little dependence on the point of entry. This notion is compatible with the findings that therapeutics aimed at different pathways (caspase activation, mitochondrial release of cytochrome c, metal binding, reactive oxygen species scavenging, etc.) all have partially salutary effects. However, it also suggests that a complete halt of the neurodegenerative process may require therapeutics that address all of the network's interacting pathways.

4. APOPTOSIS INDUCED BY MISFOLDED, UNFOLDED, OR ALTERNATIVELY FOLDED PROTEINS

One of the features common to all of the major neurodegenerative diseases is the accumulation of misfolded, unfolded, or alternatively folded proteins (Rao and Bredesen, 2004). These proteins may be the result of many different processes, such as impaired ubiquitin-mediated protein degradation, impaired chaperone-mediated autophagy or other autophagic degradative pathways, or expression of mutant proteins that may aggregate and resist proteolytic degradation. Misfolded proteins and other activators of endoplasmic reticulum (ER) stress trigger an alternative intrinsic pathway of apoptosis (Figures 12-1 and 12-2) that leads to caspase-9 activation and displays both cytochrome c/Apaf-1-independent and cytochrome c/Apaf-1-dependent activation of PCD. Cell death pathways triggered by protein misfolding, unfolding, or alternative folding and associated ER stress are of special interest in neurodegenerative disease studies, for the reason noted previously. Neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and prion protein diseases all share the common feature of ER stress. The presence of

misfolded proteins elicits cellular stress responses that include an ER stress response that serves to protect cells against the toxic accumulation of misfolded proteins and is activated by the exposure of hydrophobic protein regions that bind GRP78/BiP (glucose-regulated protein of 78 kilodaltons/binding protein), relieving its otherwise ongoing inhibition of unfolded protein response-activating proteins PERK (protein kinase R-like endoplasmic reticulum kinase), ATF6 (activating transcription factor 6), and Ire1 (inositol-requiring 1) (Figures 12-1 and 12-2). Accumulation of misfolded proteins in excessive amounts, however, overwhelms the cellular protective response that induces folding, translational, degradative, and aggresomal protection, ultimately triggering cellular suicide pathways.

Because the degradation of cellular proteins is coupled, via the ubiquitin-mediated proteasomal degradation pathway, to ER dislocation (translocating the protein targeted for degradation back out of the ER into the cytosol), any conditions that block the ER retrotranslocation of proteins or proteasome function may also result in the accumulation of misfolded protein substrates within the ER. Therefore, misfolded proteins both within and outside the ER may trigger the ER stress response. Misfolded proteins typically aggregate, initially as oligomers but ultimately as polymers that are deposited as microscopically visible inclusion bodies or plaques within cells or in extracellular spaces. These aggregates may interact with cellular targets, with several potential effects: (1) inhibition of synaptic function, (2) loss of synapses, (3) sequestration of cellular chaperones and transcription factors, (4) interference with signal transduction pathways, (5) disruption of calcium homeostasis, (6) release of free radicals, (7) dysfunction of the protein degradation pathways, and (8) induction of cell-death proteases. Despite these mechanisms, it is becoming increasingly clear that toxicity may be exerted before the appearance of aggregates, for example, with the production of small oligomers.

Mediators of PCD induced by misfolded or unfolded proteins have been identified (Figures 12-1 and 12-2). As in the classical intrinsic pathway, the Bcl-2 family proteins play a critical role in the cellular suicide decision process, communicating between the ER and the mitochondria. Bax/Bak double knockout cells fail to activate caspases after ER stress, arguing that these are required mediators. Bik may function to activate Bax and Bak in this pathway, whereas BI-1 binds to Ire1, suppressing Bax activation and translocation to the ER. Other Bcl-2 family proteins are also involved: for example, the BH3 protein Puma interacts with an hsp90 (heat shock protein of 90 kilodaltons)-independent fraction of p23, which,

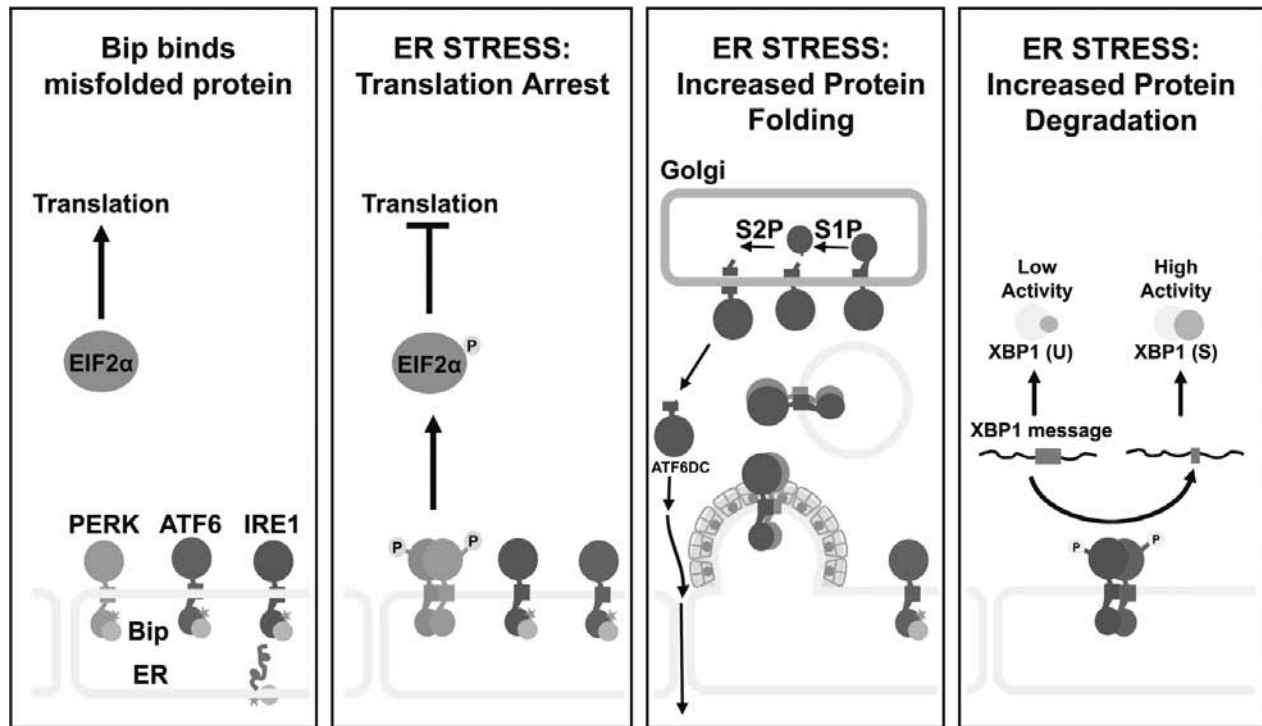


Figure 12-1. The unfolded protein response (UPR), a coordinated regulated response involving three sensor proteins: PERK (PKR-like ER kinase), ATF6 (activating transcription factor 6), and IRE1 (inositol requiring transmembrane kinase/endoribonuclease). Misfolded proteins bind Grp78/Bip, releasing it sequentially from PERK, ATF6, and IRE1. PERK undergoes oligomerization and autophosphorylation. Active PERK phosphorylates eIF2 α , rendering it inactive and blocking protein translation. Inactivation of eIF2 α prevents further influx of nascent proteins into the ER lumen, thus limiting the incoming protein load. A selective inhibitor of eIF2 α has been shown to block ER stress. Continued accumulation leads to translocation of ATF6 to the Golgi compartment where it undergoes regulated intramembrane proteolysis by proteases S1P and S2P, yielding a free cytoplasmic domain that triggers transcriptional upregulation of several ER resident proteins. These proteins facilitate and promote the productive folding of proteins and protein complexes, maintaining them in a folding-competent state and preventing their aggregation. UPR activation also induces homodimerization, autophosphorylation, and activation of IRE1, an ER resident transmembrane serine/threonine kinase receptor protein that also possesses an intrinsic endoribonuclease activity. Activated IRE1 cleaves a preformed substrate mRNA at two sites through its endoribonuclease action, resulting in the removal of a 26-nucleotide intron from a target mRNA. The two ends of the cleaved mRNA are ligated together by tRNA ligase and the newly formed mRNA encodes a transcription factor X-box binding protein (XBP-1). XBP-1 binds and activates the promoters of several ER stress-inducible target genes that facilitate retro-translocation and ER-associated degradation of misfolded proteins. IRE1 is coupled to c-Jun N-terminal kinase activation through TRAF2. Reproduced from Bredesen DE, Rao RV, Mehlen P. Cell death in the nervous system. *Nature*. Oct 19 2006;443(7113):796–802, with permission. See Color Plate 12.

when cleaved by caspases, releases Puma, leading to Bax interaction, oligomerization, and PCD. Noxa and p53 have also been implicated in this pathway.

Given the mitochondrial-ER interplay, it is not surprising that part of the resulting apoptotic pathway is Apaf-1-dependent. However, part is also Apaf-1-independent yet caspase-9-dependent. Caspase-7 is recruited to the ER by an unknown mechanism, where it interacts with caspase-12 (in the murine system; most humans do not express caspase-12, and in humans, caspase-4 may play this role); caspase-12 is cleaved and released, leading to interaction with caspase-9. Of note, murine caspase-12 lacks protease activity and plays a

predominant role as a dominant-negative inhibitor of caspase-1. GRP78/BiP interacts with caspase-7 (requiring the adenosine triphosphate [ATP]-binding domain) and -12, preventing activation, but this inhibition is relieved by (d)ATP. Although the upstream activation of this pathway is not certain, one candidate is the triggering of c-Jun N-terminal kinase activation by IRE1, via TRAF2 and ASK1.

There is also a caspase-8-dependent pathway that is activated in response to misfolded proteins: Bap31, an ER membrane protein, binds Bcl-2 (or Bcl-x_L) and a proapoptotic complex that includes caspase-8. After Bap31 cleavage, a proapoptotic p20 fragment is derived,

Thus cellular dependence on specific signals for survival is mediated, at least in part, by specific dependence receptors that induce apoptosis in the absence of the required stimulus (when unoccupied by a trophic ligand, or when bound by a competing, anti-trophic ligand), but block apoptosis after binding to their respective ligands. Expression of these dependence receptors thus

creates cellular states of dependence on the associated trophic ligands. The states of dependence are not absolute, because they can be blocked downstream in some cases by the expression of antiapoptotic genes such as Bcl-2 or p35; however, they result in a shift of the apoptotic toward an increased likelihood of triggering apoptosis. In the aggregate, these receptors may serve as a molecular integration system for trophic signals, analogous to the electrical integration system afforded by the dendritic arbors within the nervous system.

Cellular dependence on trophic signals was originally described in the developing nervous system, but does this phenomenon have anything to do with neurodegenerative disease? APP exhibits several features characteristic of dependence receptors: an intracytoplasmic caspase cleavage site (Asp664), co-immunoprecipitation with an apical caspase (caspase-8), caspase activation, derivative proapoptotic peptides (see below, this section), and suppression of apoptosis induction by mutation of the caspase cleavage site.

These findings raise several questions: first, does the caspase cleavage of APP occur in the human brain, and, if so, is this increased in patients with Alzheimer's disease? Second, if this cleavage is prevented, is the Alzheimer's phenotype affected? Third, is there a physiologic role for this cleavage event?

Neo-epitope antibodies directed against residues 657–664 of human APP disclosed the presence of caspase-cleaved APP fragments in human brain, especially in the hippocampal region. There was an approximately four-fold increase in Alzheimer's patients over age-matched controls. However, in brains without Alzheimer's pathology, there was an inverse relationship between age and immunohistochemical detection of APPneo, and the distribution was different from that of Alzheimer's disease brains. Whereas in the Alzheimer's brains the distribution was primarily in somata, in the non-Alzheimer's brains, the distribution was primarily in the processes. These findings suggest that the caspase cleavage of APP occurs physiologically and is reduced with age but is somehow increased in association with Alzheimer's disease.

The effect of preventing the caspase cleavage of APP on the Alzheimer's phenotype was evaluated in Alzheimer's disease model transgenic mice that express APP with Swedish and Indiana mutations that are associated with familial Alzheimer's disease. Although the caspase mutation (D664A) had no effect on plaque formation or on the production of A β peptides 1–40 or 1–42, the mutation prevented the synapse loss, dentate gyrus atrophy, electrophysiological abnormalities (reduction in excitatory postsynaptic potentials

and long-term potentiation), neophobia, and memory deficits that characterize Alzheimer's model mice. These findings indicate that key features of the Alzheimer's phenotype, at least in a standard transgenic mouse model, depend on the presence of the caspase cleavage site within APP. Yet extensive previous work has shown that the phenotype is also dependent on A β itself, suggesting that the APP caspase site may lie downstream from the A β accumulation. This possibility has received support from studies showing that A β interacts directly with APP in the A β region itself, leading to multimerization, caspase cleavage, and cell death signaling.

If APP does indeed function as a dependence receptor and Alzheimer's disease is a "state of altered dependence," then what is/are the trophic ligand(s) for APP? Several candidate APP interactors have been described, such as collagen (types I and IV), heparan sulfate proteoglycan, laminin, glypican, and F-spondin. In the case of F-spondin, β -secretase activity is reduced. Lourenco et al. (2009) have recently shown that netrin-1, a multifunctional axon guidance and trophic factor, also binds APP. Furthermore, netrin-1 also interacts with A β itself, and thus A β may interfere with netrin-1 binding to APP. The binding of netrin-1 to APP results in enhanced interaction of APP with Fe65 and Dab, upregulation of KAI1, and a marked reduction of net A β production (Lourenco et al., 2009).

These findings suggest a model in which the A β peptide functions as an anti-trophin, blocking netrin's guidance and trophic effects, binding and oligomerizing APP, recruiting and activating caspase-8, engendering the processing of APP at Asp664, and inducing neurite retraction, and, ultimately, neuronal cell death. An alternative possibility, however, is that the D664A mutation altered a protein–protein interaction critical to AD pathogenesis in the mouse model and that caspase cleavage is not critical. In either case, however, the results suggest that APP signal transduction may be important in mediating Alzheimer's disease, at least in the transgenic mouse model, possibly downstream from A β oligomerization and binding of APP.

The results obtained in the transgenic mouse model of AD also suggest an alternative to the classic models of AD. Chemical and physical properties of A β have been cited as the proximate cause of AD pathophysiology: reactive oxygen species generation involving A β itself, metal binding by A β , and direct membrane damage, among others (Butterfield and Bush, 2004). These theories do not explain why A β is produced ubiquitously and constitutively, nor do they offer a physiologic function for the A β peptide. They also fail to account for

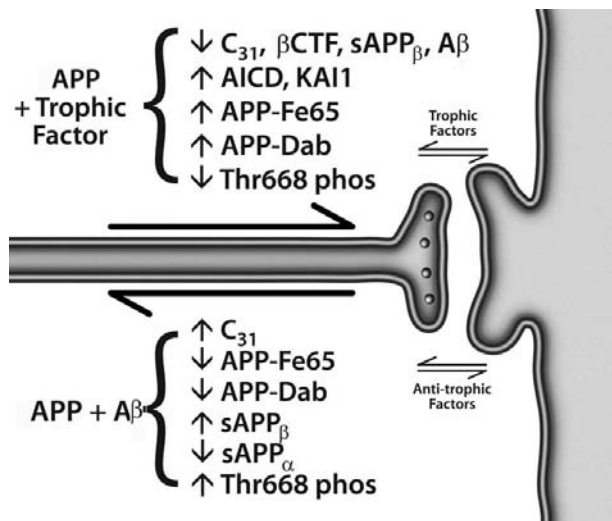


Figure 12-3. A model of Alzheimer's disease based on the concept of synaptic element interdependence mediated by APP. The presynaptic and postsynaptic elements are interdependent and provide both trophic influences (e.g., neurotrophins, netrin-1, laminin, collagen, and synaptic activity itself) and anti-trophic influences (e.g., amyloid- β peptide). Trophic support leads to the processing of APP into three peptides that support synaptic maintenance ("wholly trinity"), whereas the withdrawal of trophic support leads to alternative processing, to four peptides that mediate synaptic inhibition, synaptic loss, neurite retraction, and ultimately, programmed cell death ("four horsemen"). In this model, the A β peptide functions as an anti-trophin, and, because it leads to APP processing that produces additional A β peptide, it is "prionic" (i.e., A β begets additional A β). Reproduced from Bredesen DE, *Neurodegeneration in Alzheimer's disease: caspases and synaptic element interdependence. Mol Neurodegener.* 2009;26;4:27, with permission.

the improvement in AD model mice that occurs with a reduction in tau protein.

An alternative model, presented in Figure 12-3, argues that APP is indeed a dependence receptor and that it functions normally as a molecular switch in *synaptic element interdependence*: in this model, both the presynaptic element and the postsynaptic element are dependent on trophic support, which includes soluble factors such as netrin, substrate molecules such as laminin, neurotransmitters, and neuronal activity, as well as other factors. In the presence of adequate trophic support, APP is cleaved at the alpha and gamma sites, generating three peptides – sAPP α , p3, and APP intracellular cytoplasmic/C-terminal domain (AICD) – that support cell survival and synaptic maintenance. However, a reduction in trophic support alters the processing of APP, reducing the α/β ratio of cleavage, and leading to the production of four peptides – sAPP β , A β , Jcasp, and C31 – that mediate a reduction in synaptic transmission, synaptic loss, neurite retraction and, ultimately, programmed cell death. In this model, Alzheimer's disease is suggested to be an imbalance in physiologic

signaling pathways that mediate synaptic maintenance versus synaptic reorganization, mediated at least in part by APP, functioning in synaptic element interdependence, as part of a plasticity module that includes other receptors such as the common neurotrophin receptor, p75^{NTR}.

Caspase cleavage also appears to play an important role in cytotoxicity induced by multiple polyglutamine proteins, such as huntingtin, atrophin-1, ataxin-3, and androgen receptor. In the case of huntingtin, recruitment of caspase-2 into a complex with huntingtin was found to be polyglutamine length-dependent, leading to cleavage at Asp552 both in vitro and in vivo. Although huntingtin is not a surface receptor like APP, the upregulation of caspase-2 observed in Huntington's model mice correlated directly with decreased levels of brain-derived neurotrophic factor, suggesting that huntingtin may indeed represent a mediator of cellular dependence on trophic support. Furthermore, results analogous to those obtained with the caspase-uncleavable APP mutant described above were obtained with the caspase-6-uncleavable huntingtin mutant. In that study, the yeast artificial chromosome transgenic mouse model of Huntington's disease was used, and the Huntington's phenotype was prevented by mutating the caspase-6 cleavage site, but not by mutating the caspase-3 cleavage sites within the huntingtin protein. Altogether, these observations argue that a central component of the apoptosis machinery, caspases, play a critical role in generating the pathological fragments of APP, Huntingtin, and other toxic proteins associated with neurodegenerative diseases.

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13

Implications of Nitrosative Stress-Induced Protein Misfolding in Neurodegeneration

Tomohiro Nakamura and Stuart A. Lipton

SUMMARY

Several chronic neurodegenerative disorders manifest deposits of misfolded or aggregated proteins. Genetic mutations are the root cause for protein misfolding in rare families, but the majority of patients have sporadic forms possibly related to environmental factors. In some cases, the ubiquitin-proteasome system or molecular chaperones can prevent accumulation of aberrantly folded proteins. Recent studies suggest that generation of excessive nitric oxide (NO) and reactive oxygen species, in part due to overactivity of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor, can mediate protein misfolding in the absence of genetic mutation. S-Nitrosylation, or covalent reaction of NO with specific protein thiol groups, represents one mechanism contributing to NO-induced protein misfolding and neurotoxicity. Here, we present evidence suggesting that NO contributes to protein misfolding via S-nitrosylating protein-disulfide isomerase or the E3 ubiquitin ligase parkin. We discuss how the drugs memantine or NitroMemantine can inhibit excessive NMDA receptor activity to ameliorate NO production, protein misfolding, and neurodegeneration.

1. INTRODUCTION

Many neurodegenerative diseases are characterized by the accumulation of misfolded proteins that adversely affect neuronal connectivity and plasticity and trigger cell death signaling pathways. For example, degenerating brain contains aberrant accumulations of misfolded, aggregated proteins, such as α -synuclein and synphilin-1 in Parkinson's disease (PD) and amyloid- β (A β) and tau in Alzheimer's disease (AD). The inclusions observed in PD are called Lewy bodies and are mostly found in the cytoplasm. AD brains show intracellular neurofibrillary tangles, which contain hyperphosphorylated tau, and extracellular plaques, which contain A β . These aggregates may consist of oligomeric complexes of nonnative secondary structures and demonstrate poor solubility in aqueous or detergent solvent. Other disorders manifesting protein aggregation include Huntington's disease

(a polyQ disorder), amyotrophic lateral sclerosis (ALS), and prion disease.

An additional feature of most neurodegenerative diseases is excessive generation of reactive nitrogen species (RNS) and reactive oxygen species (ROS), which can contribute to neuronal cell injury and death. Although many intra- and extracellular molecules may participate in neuronal injury, accumulation of nitrosative stress due to excessive generation of nitric oxide (NO) appears to be a potential factor contributing to neuronal cell damage and death. A well-established model for NO production entails a central role of the *N*-methyl-D-aspartate (NMDA)-type glutamate receptors in the nervous system. Excessive activation of NMDA receptors drives Ca²⁺ influx, which in turn activates neuronal NO synthase (nNOS), as well as the generation of ROS (Figure 13-1). Accumulating evidence suggests that NO can mediate both protective and neurotoxic effects by reacting with cysteine residues of target proteins to form S-nitrosothiols (SNOs), a process termed S-nitrosylation because of its effects on the chemical biology of protein function. Importantly, normal mitochondrial respiration may also generate free radicals, principally ROS, and one such molecule, superoxide anion (O₂⁻), reacts rapidly with free radical NO to form the very toxic product peroxynitrite (ONOO⁻).

Importantly, protein aggregation can result from either (1) a rare mutation in the disease-related gene encoding the protein, or (2) post-translational changes to the protein engendered by nitrosative/oxidative stress, which may well account for the more common sporadic cases of the disease. Therefore, a key theme of this article is the hypothesis that nitrosative and oxidative stress contribute to protein misfolding in the brains of the majority of neurodegenerative patients. In this review, we discuss specific examples showing that

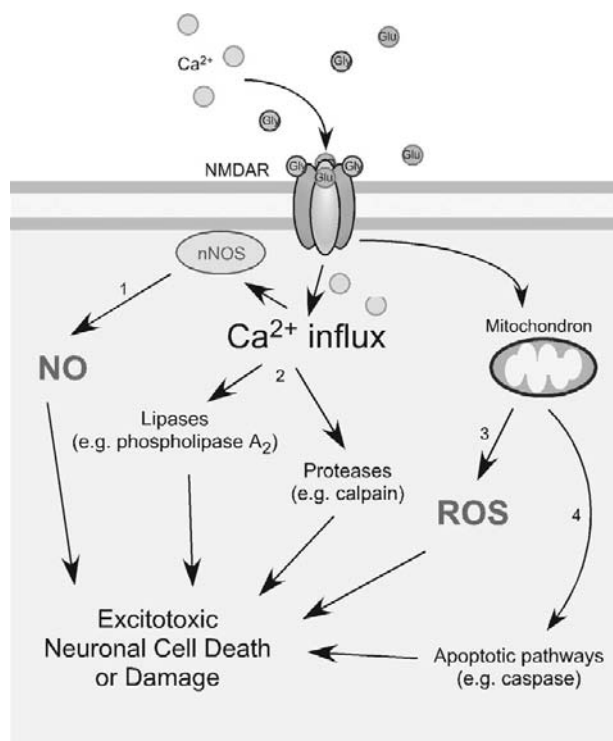


Figure 13-1. Activation of the NMDA receptor (NMDAR) by glutamate (Glu) and glycine (Gly) induces Ca^{2+} influx and activates excitotoxic pathways. NMDAR hyperactivation triggers (1) generation of NO, (2) activation of lipases and proteases, (3) production and release of ROS from mitochondria, and (4) activation of caspases, contributing to neuronal cell death and damage.

S-nitrosylation of (1) ubiquitin E3 ligases such as parkin or (2) endoplasmic reticulum chaperones such as protein-disulfide isomerase (PDI) is critical for the accumulation of misfolded proteins in neurodegenerative diseases such as PD and other conditions. We also discuss the neuroprotective mechanism of action of NMDA open-channel blockers like memantine and NO-related drugs for the treatment of neurodegenerative disorders.

2. PROTEIN MISFOLDING AND AGGREGATION IN NEURODEGENERATIVE DISEASES

In general, protein aggregates do not accumulate in unstressed, healthy neurons due in part to the existence of cellular “quality control machineries.” For example, molecular chaperones are believed to provide a defense mechanism against the toxicity of misfolded proteins because chaperones can prevent inappropriate interactions within and between polypeptides and can promote refolding of proteins that have been misfolded because of cell stress. In addition to the quality control of proteins provided by molecular chaperones, the ubiquitin–proteasome system (UPS) and autophagy/lysosomal degradation are involved in the clearance of abnormal

or aberrant proteins. When chaperones cannot repair misfolded proteins, they may be tagged via addition of polyubiquitin chains for degradation by the proteasome. In neurodegenerative conditions, intra- or extracellular protein aggregates are thought to accumulate in the brain as a result of a decrease in molecular chaperone or proteasome activities. In fact, several mutations that disturb the activity of molecular chaperones or UPS-associated enzymes can cause neurodegeneration. Along these lines, postmortem samples from the substantia nigra of PD patients (vs. non-PD controls) manifest a significant reduction in proteasome activity.

Historically, lesions that contain aggregated proteins were considered to be pathogenic. Recently, several lines of evidence have suggested that aggregates are formed through a complex multistep process by which misfolded proteins assemble into inclusion bodies; currently, soluble (micro-)oligomers of these aberrant proteins are thought to be the most toxic forms via interference with normal cell activities, whereas frank macroscopic aggregates may be an attempt by the cell to wall off potentially toxic material.

3. NMDA RECEPTOR-MEDIATED GLUTAMATERGIC SIGNALING PATHWAYS INDUCE Ca^{2+} INFLUX AND GENERATION OF RNS/ROS

It is well known that the amino acid glutamate is the major excitatory neurotransmitter in the brain. Glutamate is present in high concentrations in the adult central nervous system and is released for milliseconds from nerve terminals in a Ca^{2+} -dependent manner. After glutamate enters the synaptic cleft, it diffuses across the cleft to interact with its corresponding receptors on the postsynaptic face of an adjacent neuron. Excitatory neurotransmission is necessary for the normal development and plasticity of synapses and for some forms of learning or memory; however, excessive activation of glutamate receptors is implicated in neuronal damage in many neurological disorders, ranging from acute hypoxic-ischemic brain injury to chronic neurodegenerative diseases. John Olney coined the term *excitotoxicity* to describe the toxic effect of glutamate. It is currently thought that overstimulation of extrasynaptic NMDA receptors mediate, at least in part, this type of neuronal damage, whereas, in contrast, synaptic activity predominantly activates survival pathways. Intense hyperstimulation of excitatory receptors leads to necrotic cell death, but more mild or chronic overstimulation can result in apoptotic or other forms of cell death.

There are two large families of glutamate receptors in the nervous system, ionotropic receptors (representing

ligand-gated ion channels) and metabotropic receptors (coupled to G-proteins). Ionotropic glutamate receptors are further divided into three broad classes: (1) NMDA receptors, (2) α -amino-3-hydroxy-5 methyl-4-isoxazole propionic acid (AMPA) receptors, and (3) kainate receptors, which are each named after synthetic ligands that can selectively activate these receptors. The NMDA receptor has attracted attention for a long period of time because it has several properties that set it apart from other ionotropic glutamate receptors. One such characteristic, in contrast to most AMPA and kainate receptors, is that NMDA receptor-coupled channels are highly permeable to Ca^{2+} , thus permitting Ca^{2+} entry after ligand binding if the cell is depolarized to relieve block of the receptor-associated ion channel by Mg^{2+} . Subsequent binding of Ca^{2+} to various intracellular molecules can lead to many significant consequences. For instance, increased levels of neuronal Ca^{2+} , in conjunction with the Ca^{2+} -binding protein CaM, trigger the activation of nNOS and subsequent generation of NO from the amino acid L-arginine. NO is a gaseous free radical (thus highly diffusible) and a key molecule that plays a vital role in normal signal transduction, but in excess it can lead to neuronal cell damage and death. In particular, excessive activation of NMDA receptors leads to the production of damaging free radicals (e.g., NO and ROS) and other enzymatic processes, contributing to cell death (Figure 13-1).

Increased nitrosative and oxidative stress are associated with chaperone and proteasomal dysfunction, resulting in accumulation of misfolded aggregates. However, until recently, little was known regarding the molecular and pathogenic mechanisms underlying contribution of NO to the formation of inclusion bodies such as amyloid plaques in AD or Lewy bodies in PD.

4. PROTEIN S-NITROSYLATION AND NEURONAL CELL DEATH

Early investigations indicated that NO participates in cellular signaling pathways, which regulate broad aspects of brain function, including synaptic plasticity, normal development, and neuronal cell death. In general, NO exerts physiologic and some pathophysiologic effects via stimulation of guanylate cyclase to form cyclic guanosine-3',5'-monophosphate or through S-nitros(yl)ation of regulatory protein thiol groups. S-Nitrosylation is the covalent addition of an NO group to a critical cysteine thiol/sulfhydryl (or, more properly, thiolate anion, RS^-) to form an S-nitrosothiol derivative (R-SNO). Such modification modulates the function of a broad spectrum of mammalian, plant, and microbial

proteins. In general, a consensus motif of amino acids comprised of nucleophilic residues (generally an acid and a base) surround a critical cysteine, which increases the cysteine sulfhydryl's susceptibility to S-nitrosylation. Our group first identified the physiologic relevance of S-nitrosylation by showing that NO and related RNS exert paradoxical effects via redox-based mechanisms – NO is neuroprotective via S-nitrosylation of NMDA receptors (as well as other subsequently discovered targets, including caspases) and yet can also be neurodestructive by formation of peroxynitrite (or, as later discovered, reaction with additional molecules such as matrix metalloproteinase 9 and glyceraldehyde 3-phosphate dehydrogenase [GAPDH]). Over the past decade, accumulating evidence has suggested that S-nitrosylation can regulate the biological activity of a great variety of proteins, in some ways akin to phosphorylation. Chemically, NO is often a good “leaving group,” facilitating further oxidation of critical thiol to disulfide bonds among neighboring (vicinal) cysteine residues or, via reaction with ROS, to sulfenic (-SOH), sulfinic (-SO₂H) or sulfonic (-SO₃H) acid derivitization of the protein.

Inhibition of NOS activity ameliorates the progression of disease pathology in animal models of PD, AD, and ALS, suggesting that excess generation of NO plays a pivotal role in the pathogenesis of several neurodegenerative diseases. Although the involvement of NO in neurodegeneration has been widely accepted, the chemical relationship between nitrosative stress and accumulation of misfolded proteins has remained obscure. Recent findings, however, have shed light on molecular events underlying this relationship. Specifically, we recently mounted physiologic and chemical evidence that S-nitrosylation modulates the (1) ubiquitin E3 ligase activity of parkin, and (2) chaperone and isomerase activities of PDI, contributing to protein misfolding and neurotoxicity in models of neurodegenerative disorders (Figure 13-2).

5. S-NITROSYLATION OF PARKIN

Identification of errors in the genes encoding parkin (a ubiquitin E3 ligase) and UCH-L1 (deubiquitinating enzyme) in rare familial forms of PD has implicated possible dysfunction of the UPS in the pathogenesis of sporadic PD. The UPS represents an important mechanism for proteolysis in mammalian cells. Formation of polyubiquitin chains constitutes the signal for proteasomal attack and degradation. An isopeptide bond covalently attaches the C-terminus of the first ubiquitin in a polyubiquitin chain to a lysine residue in the target protein. The cascade of activating (E1), conjugating (E2),

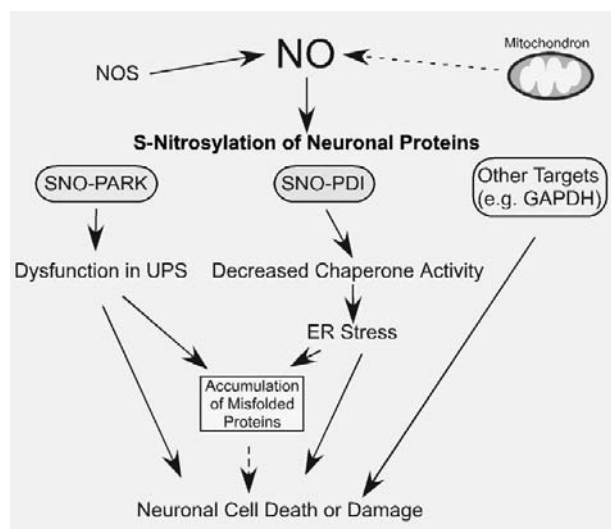


Figure 13-2. Possible mechanism whereby S-nitrosylated species contribute to the accumulation of aberrant proteins and neuronal damage. S-nitrosylation of parkin (forming SNO-PARK) and PDI (forming SNO-PDI) can contribute to neuronal cell injury in part by triggering accumulation of misfolded proteins. S-Nitrosylation of other proteins, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), may also contribute to neuronal cell injury or death.

and ubiquitin-ligating (E3) type enzymes catalyzes the conjugation of the ubiquitin chain to proteins. In addition, individual E3 ubiquitin ligases play a key role in the recognition of specific substrates.

Mutations in the parkin gene can cause autosomal-recessive juvenile Parkinsonism (ARJP), accounting for some cases of hereditary PD manifest in young patients with onset beginning anywhere from the teenage years through the 40s. Parkin is a member of a large family of E3 ubiquitin ligases that are related to one another by the presence of RING (really interesting new gene) finger domains. Point mutations, stop mutations, truncations, and deletions in both alleles of the parkin gene will eventually cause dysfunction in its activity and are responsible for many cases of ARJP as well as rare adult forms of PD.

Nitrosative/oxidative stress, commonly found during normal aging, can mimic rare genetic causes of disorders, such as PD, by promoting protein misfolding in the absence of a genetic mutation. For example, S-nitrosylation and further oxidation of parkin or Uch-L1 result in dysfunction of these enzymes and thus of the UPS (Figure 13-2). We and others recently discovered that nitrosative stress triggers S-nitrosylation of parkin (forming SNO-parkin) not only in rodent models of PD, but also in the brains of human patients with PD and the related α -synucleinopathy, diffuse Lewy body disease. SNO-parkin initially stimulates ubiquitin E3 ligase activity, resulting in enhanced ubiquitination as observed in

Lewy bodies, followed by a decrease in enzyme activity, producing a futile cycle of dysfunctional UPS (Figure 13-2). We also found that rotenone led to the generation of SNO-parkin and thus dysfunctional ubiquitin E3 ligase activity. Moreover, S-nitrosylation appears to compromise the neuroprotective effect of parkin. These mechanisms involve S-nitrosylation of critical cysteine residues in the first RING domain of parkin. Nitrosative and oxidative stress can also alter the solubility of parkin via post-translational modification of cysteine residues, which may concomitantly compromise its protective function.

6. S-NITROSYLATION OF PDI MEDIATES PROTEIN MISFOLDING AND NEUROTOXICITY IN CELL MODELS OF PD OR AD

The endoplasmic reticulum (ER) normally participates in protein processing and folding but undergoes a stress response when immature or misfolded proteins accumulate. ER stress stimulates two critical intracellular responses. The first represents expression of chaperones that prevent protein aggregation via the unfolded protein response (UPR) and is implicated in protein refolding, post-translational assembly of protein complexes, and protein degradation. The second ER stress response, termed ER-associated degradation, specifically recognizes terminally misfolded proteins for retrotranslocation across the ER membrane to the cytosol, where they can be degraded by the UPS. Additionally, although severe ER stress or a prolonged UPR can induce apoptosis, the ER withstands relatively mild insults via expression of stress proteins such as glucose-regulated protein and PDI. These proteins behave as molecular chaperones that assist in the maturation, transport, and folding of secretory proteins.

During protein folding in the ER, PDI can also introduce disulfide bonds into proteins (oxidation), break disulfide bonds (reduction), and catalyze thiol/disulfide exchange (isomerization), thus facilitating disulfide bond formation, rearrangement reactions, and structural stability. PDI has two redox active CXXC motifs, and these two-thiol/disulfide centers function as independent active sites.

In many neurodegenerative disorders and cerebral ischemia, the accumulation of immature and denatured proteins results in ER dysfunction, but upregulation of PDI represents an adaptive response promoting protein refolding and may offer neuronal cell protection. In addition, it is generally accepted that excessive generation of NO can contribute to activation of the ER stress pathway, at least in some cell types. Molecular mechanisms

by which NO induces protein misfolding and ER stress, however, have remained enigmatic until recently.

Interestingly, we have recently reported that excessive NO can also lead to S-nitrosylation of the active-site thiol groups of PDI, and this reaction inhibits both its isomerase and chaperone activities (Figure 13-2). Mitochondrial complex I insult by rotenone can also result in S-nitrosylation of PDI in cell culture models. Moreover, we found that PDI is S-nitrosylated in the brains of virtually all cases examined of sporadic AD and PD. Under pathological conditions, it is possible that both cysteine sulfhydryl groups in the active sites of PDI form S-nitrosothiols. Additionally, we speculate that vicinal (nearby) cysteine thiols reacting with NO can form nitroxyl disulfide, and such reaction may potentially occur in the catalytic side of PDI to inhibit enzymatic activity. To determine the consequences of S-nitrosylated PDI (SNO-PDI) formation in neurons, we exposed cultured cerebrocortical neurons to neurotoxic concentrations of NMDA, thus inducing excessive Ca^{2+} influx and consequent NO production from nNOS. Under these conditions, we found that PDI was S-nitrosylated in a NOS-dependent manner. SNO-PDI formation led to the accumulation of polyubiquitinated/misfolded proteins and activation of the UPR. Moreover, S-nitrosylation abrogated the inhibitory effect of PDI on aggregation of proteins observed in Lewy body inclusions. S-Nitrosylation of PDI also prevented its attenuation of neuronal cell death triggered by ER stress, misfolded proteins, or proteasome inhibition (Figure 13-2).

The UPS and UPR are apparently impaired in the aging brain. Additionally, inclusion bodies similar to those found in neurodegenerative disorders can appear in brains of normal aged individuals or those with sub-clinical manifestations of disease. These findings suggest that the activity of the UPS and molecular chaperones may decline in an age-dependent manner. Given that we have not found detectable quantities of SNO-parkin and SNO-PDI in normal aged brain, we speculate that S-nitrosylation of these and similar proteins may represent a key event that contributes to susceptibility of the aging brain to neurodegenerative conditions.

7. POTENTIAL TREATMENT OF EXCESSIVE NMDA-INDUCED Ca^{2+} INFLUX AND FREE RADICAL GENERATION

One mechanism that could potentially curtail excessive Ca^{2+} influx and resultant overstimulation of nNOS activity, with resultant S-nitrosylation of parkin, PDI, and other proteins, would be inhibition of NMDA receptors. Until recently, however, drugs in this class blocked

virtually all NMDA receptor activity, including physiological activity, and therefore manifest unacceptable side effects by inhibiting normal functions of the receptor. For this reason, many previous NMDA receptor antagonists have disappointingly failed in advanced clinical trials conducted for a number of neurodegenerative disorders. In contrast, studies in our laboratory first showed that the adamantane derivative, memantine, preferentially blocks excessive (pathological) NMDA receptor activity while relatively sparing normal (physiologic) activity. Memantine does this in a surprising fashion because of its low (micromolar) affinity, even though its actions are quite selective for the NMDA receptor at that concentration (Figure 13-3). “Apparent” affinity of a drug is determined by the ratio of its “on-rate” to its “off-rate” for the target. The on-rate is not only a property of drug diffusion and interaction with the target, but also the drug’s concentration. In contrast, the off-rate is an intrinsic property of the drug-receptor complex, unaffected by drug concentration. A relatively fast off-rate is a major contributor to memantine’s low affinity for the NMDA receptor. The inhibitory activity of memantine involves blockade of the NMDA receptor-associated ion channel when it is excessively open (termed *open-channel block*). The unique and subtle difference of the memantine blocking sites in the channel pore may explain the advantageous properties of memantine action.

Also critical for the clinical tolerability of memantine is its uncompetitive mechanism of action. An uncompetitive antagonist can be distinguished from a non-competitive antagonist, which acts allosterically at a noncompetitive site (i.e., at a site other than the agonist-binding site). An uncompetitive antagonist is defined as an inhibitor whose action is contingent on prior activation of the receptor by the agonist. Hence the same amount of antagonist blocks higher concentrations of agonist relatively better than lower concentrations of agonist. Some open-channel blockers function as pure uncompetitive antagonists, depending on their exact properties of interaction with the ion channel. This uncompetitive mechanism of action coupled with a relatively fast off-rate from the channel yields a drug that preferentially blocks NMDA receptor-operated channels when they are excessively open while relatively sparing normal neurotransmission. In fact, the relatively fast off-rate is a major contributor to a drug like memantine’s low affinity for the channel pore. Although many factors determine a drug’s clinical efficacy and tolerability, it appears that the relatively rapid off-rate is a predominant factor in memantine’s tolerability in contrast to other NMDA-type receptor antagonists. Thus the

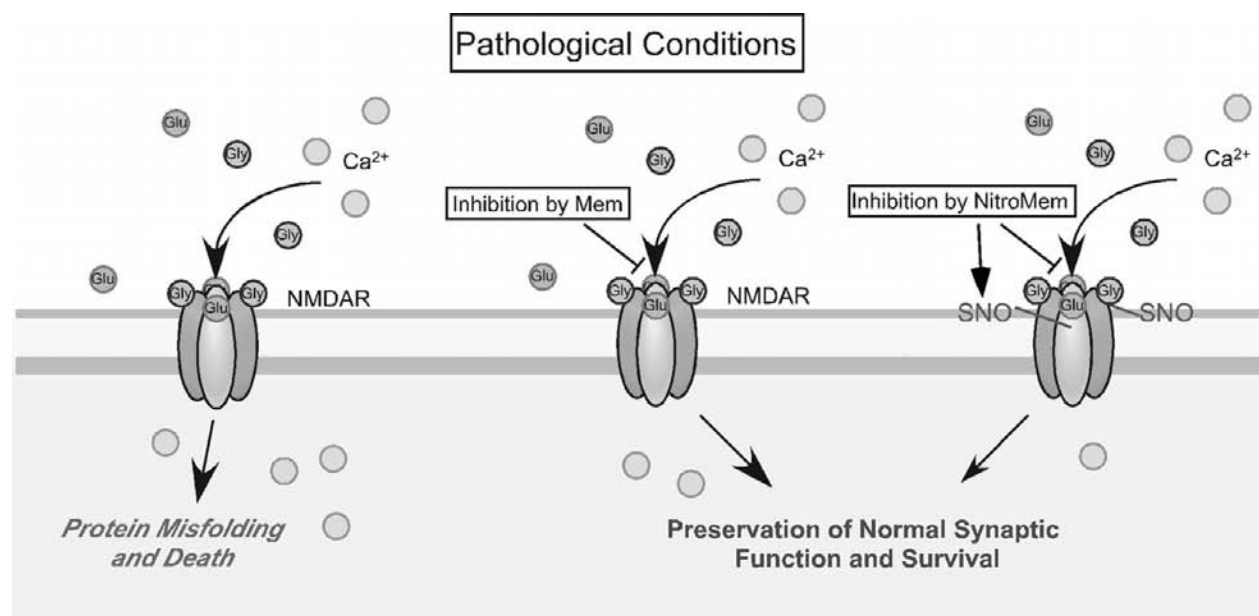


Figure 13-3. Memantine and NitroMemantine preferentially block excessive extrasynaptic NMDA receptor activity. (Left) Excessive activation of the NMDA receptor (NMDAR), predominantly at extrasynaptic sites, is thought to induce neuronal cell injury and death and is associated with the accumulation of misfolded proteins. (Middle and Right) Memantine (Mem) and the newer NitroMemantines (NitroMem) preferentially block excessive (pathological extrasynaptic) NMDA receptor activity while relatively sparing normal (physiologic synaptic) activity. (Right) NitroMemantines target an NO species to the NMDA receptor by tethering a nitro group to the memantine moiety and thus add to the neuroprotective action of memantine by S-nitrosylating the receptor.

critical features of memantine's mode of action are its *Uncompetitive* mechanism and *Fast Off-rate*, or what we call a UFO drug – a drug that is present at its site of inhibitory action only when you need it and then quickly disappears. Memantine has been used for many years in Europe to treat PD, and regulatory agencies in both Europe and the United States recently voted its approval as the first treatment for moderate-to-severe AD. It is currently under study for a number of other neurodegenerative disorders.

As promising as the results with memantine are, we are continuing to pursue ways to use additional modulatory sites on the NMDA receptor to block excitotoxicity even more effectively and safely than memantine alone. New approaches in this regard are explored next.

8. FUTURE THERAPEUTICS: NITROMEMANTINES

NitroMemantines are second-generation memantine derivatives that are designed to have enhanced neuroprotective efficacy without sacrificing clinical tolerability. As mentioned earlier, a nitrosylation site(s) is located on the extracellular domain of the NMDA receptor, and S-nitrosylation of this site (i.e., NO reaction with the sulfhydryl group of a critical cysteine residue) down-regulates (but does not completely shut off) receptor

activity (Figure 13-3). The drug nitroglycerin, which generates NO-related species, can act at this site to limit excessive NMDA receptor activity. In fact, in rodent models, nitroglycerin can limit ischemic damage, and there is some evidence that patients taking nitroglycerin for other medical reasons may be resistant to glaucomatous visual field loss. Consequently, we carefully characterized the S-nitrosylation sites on the NMDA receptor to determine whether we could design a nitroglycerin-like drug that could be more specifically targeted to the receptor. In brief, we found that five different cysteine residues on the NMDA receptor could interact with NO. One of these, located at cysteine residue 399 (Cys399) on the NR2A subunit of the NMDA receptor, mediates $\geq 90\%$ of the effect of NO under our experimental conditions. From crystal structure models and electrophysiologic experiments, we further found that NO binding to the NMDA receptor at Cys399 may induce a conformational change in the receptor protein that makes glutamate and Zn²⁺ bind more tightly to the receptor. The enhanced binding of glutamate and Zn²⁺ in turn causes the receptor to desensitize and, consequently, the ion channel to close. Electrophysiologic studies have demonstrated this inhibitory effect of NO on the NMDA receptor-associated channel. Moreover, as the oxygen tension is lowered (a pO₂ of 10–20 torr is found in

normal brain, and even lower levels under hypoxic/ischemic conditions), the NMDA receptor becomes more sensitive to inhibition by S-nitrosylation.

Unfortunately, nitroglycerin itself is not very attractive as a neuroprotective agent. The same cardiovascular vasodilator effect that makes it useful in the treatment of angina could cause dangerously large drops in blood pressure in patients with dementia, stroke, traumatic injury, or glaucoma. However, the open-channel block mechanism of memantine not only leads to a higher degree of channel blockade in the presence of excessive levels of glutamate, but also can be used as a homing signal for targeting drugs (e.g., the NO group) to hyper-activated, open NMDA-gated channels. We have therefore been developing combinatorial drugs (NitroMemantines) that theoretically should be able to use memantine to target NO to the nitrosylation sites of the NMDAR to avoid the systemic side effects of NO. Two sites of modulation would be analogous to having two volume controls on your television set for fine-tuning the audio signal.

Preliminary studies have shown NitroMemantines to be highly neuroprotective in both in vitro and in vivo animal models. In fact, they appear to be more effective than memantine at lower dosage. Moreover, because of the targeting effect of the memantine moiety, NitroMemantines appear to lack the blood pressure-lowering effects typical of nitroglycerin. More research still needs to be performed on NitroMemantine drugs, but by combining two clinically tolerated drugs (memantine and nitroglycerin), we have created a new, improved class of UFO drugs that should be both clinically tolerated and neuroprotective.

9. CONCLUSIONS

Excessive nitrosative and oxidative stress triggered by excessive NMDA receptor activation and/or mitochondrial dysfunction may result in malfunction of the UPS or molecular chaperones, thus contributing to abnormal protein accumulation and neuronal damage in sporadic forms of neurodegenerative diseases. Our elucidation of an NO-mediated pathway to dysfunction of parkin and PDI by S-nitrosylation provides a mechanistic link between free radical production, abnormal protein accumulation, and neuronal cell injury in neurodegenerative disorders such as PD. Elucidation of this new pathway may lead to the development of additional new therapeutic approaches to prevent aberrant protein misfolding by targeted disruption or prevention of nitrosylation of specific proteins such as parkin and PDI. This article also describes the action of memantine

via uncompetitive antagonism of the NMDA receptor with a fast off-rate. NitroMemantines enhance the neuroprotective efficacy over memantine at a given dose owing to its additional ability to S-nitrosylate the NMDA receptor. These drugs preferentially inhibit pathologically activated NMDA receptor while preserving its normal synaptic function; thus they are clinically tolerated. In this chapter we propose that the next generation of CNS drugs will interact with their target only during states of pathological activation and not interfere with the target if it is functioning properly. In the future, such perspectives should lead to additional novel, clinically tolerated neuroprotective therapeutics.

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Mitochondrial Mechanisms of Neural Cell Death in Cerebral Ischemia

Lucian Soane, Brian M. Polster, and Gary Fiskum

1. CELL DEATH AFTER CEREBRAL ISCHEMIA AND REPERFUSION

Millions die annually from ischemic brain damage caused by stroke, subarachnoid hemorrhage, head trauma, shock, and postischemic injury after resuscitation from cardiac arrest. Many thousands of others either die or suffer permanent neurological impairment after surgical procedures that carry a risk for inducing cerebral hypoxia/ischemia. Neurological morbidity and mortality are primarily the consequences of necrotic, apoptotic, and other forms of neuronal and nonneuronal cell death. The most effective neuroprotective interventions must therefore target mechanisms that contribute to each of these pathways.

Traditionally, brain cell death after cerebral ischemia has been considered to be primarily necrotic in nature. More recent research revealed, however, that the pattern of cell death in the postischemic brain is much more complex. On the basis of morphology alone, both necrotic and apoptotic death mechanisms and (according to recent studies) autophagy seem to contribute to some extent to the demise of injured cells. The extent of contribution of each of these mechanisms depends on the intensity and type of injury, whether it is due to focal or transient global cerebral ischemia, the brain region(s) affected, time post-injury, and so forth. Although necrosis predominates in the center of a focal ischemic lesion (ischemic core) and can occur early (within minutes), cells with apoptotic morphology are generally reported in the penumbral region and peak at later stages (days, even weeks). In contrast, initiation of apoptotic molecular pathways has been observed within 30 minutes of reperfusion after global cerebral ischemia caused by cardiac arrest, even though most neurons that are dead or dying 24 hours later appear

necrotic. A similar course of events has been described for the core region of ischemic strokes. It has been noted, however, that in the postischemic brain, dying neurons often assume morphologies that do not reflect purely apoptotic or necrotic characteristic features, but incomplete (apoptotic- or necrotic-like) or mixed phenotypes. Accordingly, some studies have proposed the existence of a necrosis-apoptosis continuum. Multiple cell death mechanisms can be activated in the injured cells, and in some models even within the same cells. The phenotype acquired by the dying cells reflects thus the degree of completion of these death pathways (i.e., availability of adenosine triphosphate [ATP] is required for completion of apoptosis). It has been suggested that a process of secondary necrosis results from rapid failure to fully develop the apoptotic pathways due to rapid depletion of energy stores. Such shifts from apoptosis to necrosis have also been demonstrated in *in vitro* models, and many studies begin to reveal the existence of extensive cross-communication between various cell death pathways. Although necrosis has been considered for a long time just as an accidental, unregulated form of cell death, there is now substantial evidence indicating that similar to other death pathways (i.e., apoptosis), execution of at least certain forms of necrosis is also highly regulated. Several specific mediators of necrotic death have been characterized, including RIP1 kinase induced by death receptor (DR) ligands, poly-(ADP-ribose) polymerase (PARP) 1 overactivation, and multiple mitochondrial alterations (i.e., mitochondrial permeability transition [MPT], reactive oxygen species [ROS] production; see next section) (Figure 14-1). The term *necroptosis* has been proposed to distinguish the regulated necrotic death from accidental necrosis. A central role in coordinating many of the cell death pathways implicated in ischemia/reperfusion is held by mitochondria.

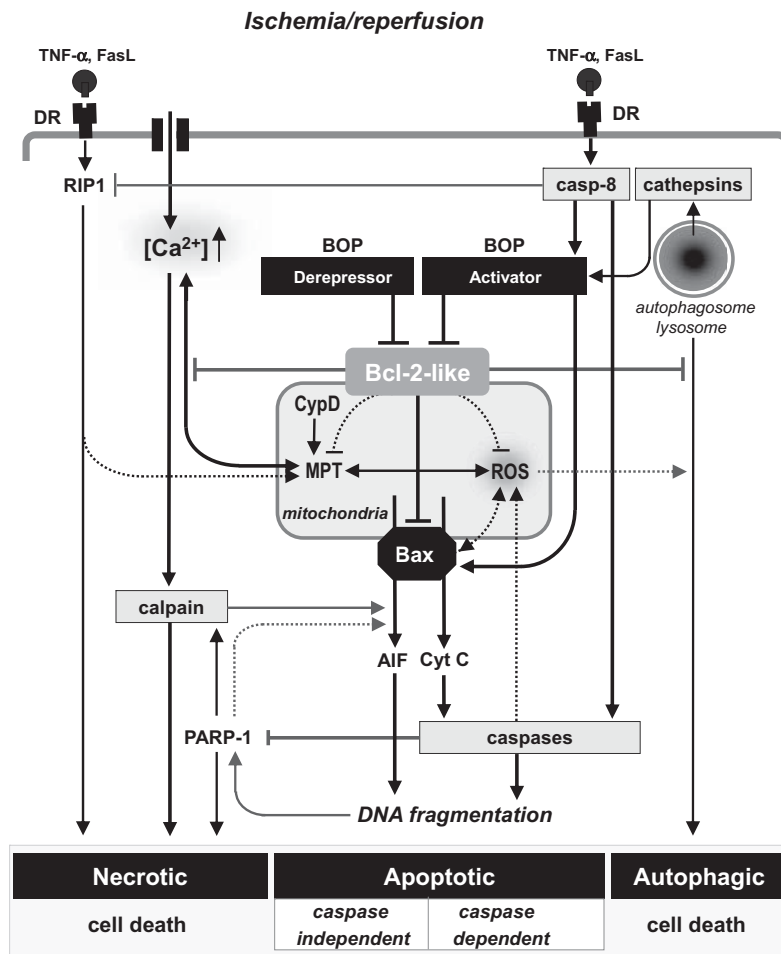


Figure 14-1. Cell death pathways contributing to ischemic brain injury. Antiapoptotic (Bcl-2-like) and proapoptotic Bcl-2 family proteins, including multidomain proteins (Bax) and the BH3-only (BOP) protein subgroup (divided into activators and de-repressors), are central regulators of the intrinsic mitochondrial apoptotic pathway that control the release of apoptogenic proteins from the mitochondrial intermembrane space into the cytosol. Apoptogenic mitochondrial proteins trigger cell death in a caspase-dependent (e.g., Cyt C) or -independent manner (e.g., AIF). Excitotoxic mechanisms resulting in massive $[Ca^{2+}]_{ic}$ influx and calpain activation, PARP-1 overactivation leading to depletion of NADH pools affecting mitochondrial bioenergetic and metabolic functions, and RIP1 activation by DR ligands (i.e., tumor necrosis factor alpha) result in necrotic (or necrotic-like) cell death. Cross-communications exist between cell death pathways (gray lines; see text for details). Bcl-2-like proteins can interfere with both autophagic death through binding to the autophagy regulator beclin-1 and necrotic death through regulation of various mitochondrial processes, including mitochondrial Ca^{2+} uptake capacity, MPT, and redox state. Further cross-communication occurs at later stages through the activities of calpains and caspases.

Mitochondria are also responsible for executing early steps in apoptosis through their release of proteins (e.g., cytochrome c and apoptosis-inducing factor [AIF]) that acquire toxic functions in the cytosol or nucleus (Figure 14-2). In general, relatively mild mitochondrial injury results primarily in apoptotic cell death, whereas more extensive injury leads to necrosis as a result of metabolic failure.

Excessive neuronal accumulation of Ca^{2+} that occurs during excitotoxic stimulation and during ischemia/reperfusion is not the only mediator of mitochondrial injury. Mitochondria are highly sensitive targets of ROS generated by several systems, including the mitochondrial electron transport chain, cyclooxygenases, Fe^{2+} -catalyzed formation of hydroxyl radicals from hydrogen peroxide, NAD(P)H oxidases, and peroxynitrite formed from the reaction of nitric oxide with superoxide. These species and the influence that their metabolism has on mitochondrial redox state result in oxidative modification of proteins, DNA, RNA, and membrane lipids. Any of these modifications can affect the rate or energy coupling efficiency of oxidative phosphorylation. Oxidation of protein amino acids, including that due to tyrosine nitration and cysteine S-nitrosylation, is particularly prevalent during ischemia/reperfusion and appears responsible for inhibition of energy transduction both at the level of matrix enzymes (e.g., pyruvate dehydrogenase) and at the level of the electron transport chain.

2. MITOCHONDRIA MEDIATE BOTH NECROTIC AND APOPTOTIC CELL DEATH

Mitochondria have long been considered as subcellular targets of ischemic brain injury. The significance of ischemic mitochondrial injury was initially thought to be limited to the effects on maintaining sufficient cellular ATP levels to avoid necrotic cell death. Mitochondria were subsequently characterized as a major source of toxic ROS, contributing to acute excitotoxic neuronal death in response to elevated intracellular Ca^{2+} .

3. MITOCHONDRIAL PERMEABILITY TRANSITION ACTIVATED BY Ca^{2+} AND OXIDATIVE STRESS

The combined exposure of mitochondria to high Ca^{2+} plus oxidative stress is far more toxic than either stress alone. The primary target for this pathologic synergism is the inner membrane permeability transition pore (PTP), which is responsible for initiating the MPT. The MPT is defined as a relatively nonspecific increase in inner membrane permeability to solutes $\leq 1,500$ Da that results

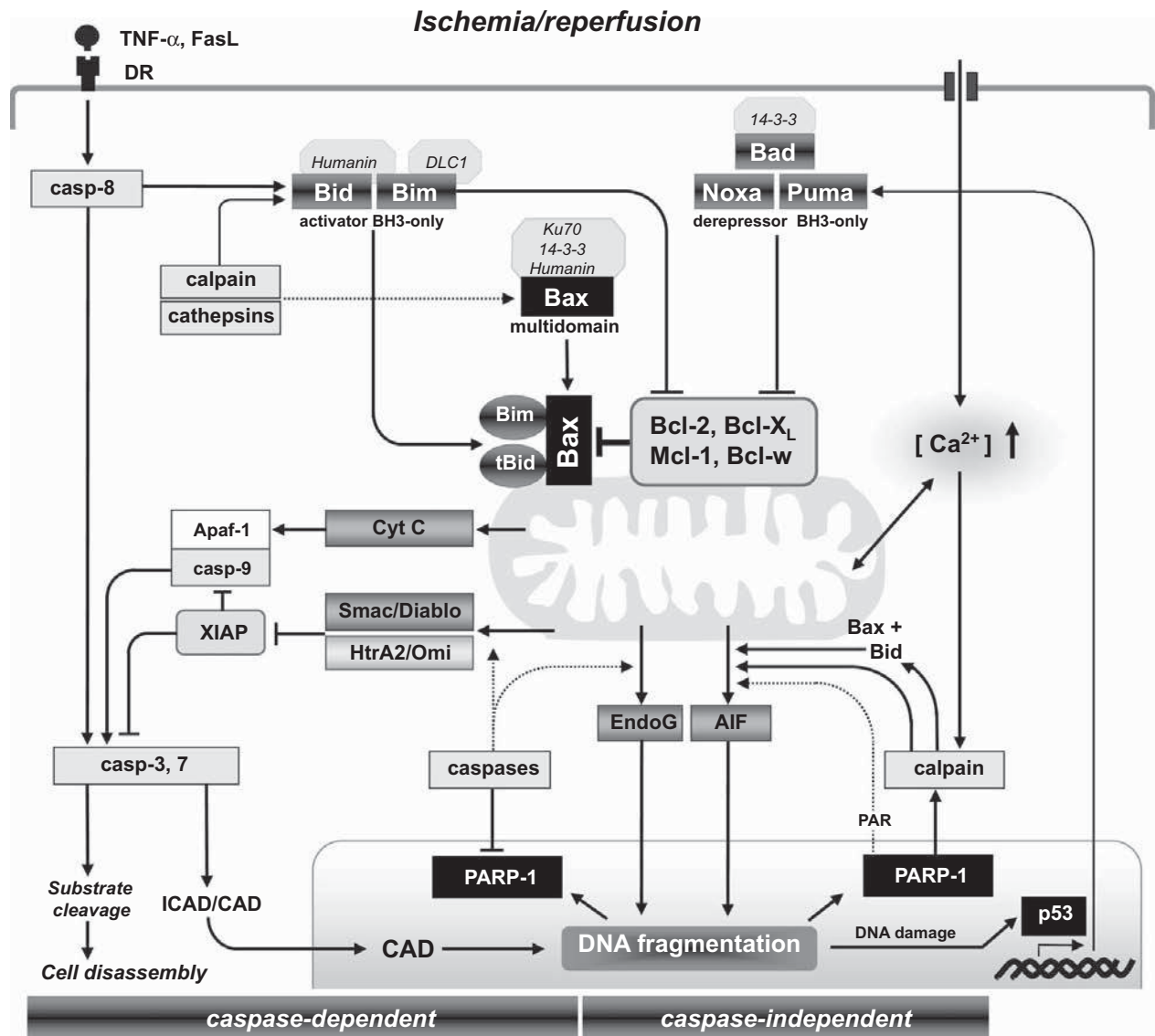


Figure 14-2. Schematic representation of the mitochondrial intrinsic apoptotic pathway. See text for details.

in osmotic swelling of the mitochondrial matrix and collapse of the mitochondrial membrane potential. In addition to uncoupling oxidative phosphorylation, PTP opening allows for release of critical small molecules from the matrix, including NAD(P)H and glutathione. Moreover, high-amplitude osmotic swelling results in rupture or breakage of the relatively inflexible outer membrane, releasing cytochrome c, a peripheral membrane protein component of the respiratory chain, into the cytosol. Mitochondria that undergo these extreme consequences of PTP opening are irreversibly damaged and only contribute to ATP hydrolysis, rather than ATP production. Depending on the tissue or cell type, PTP opening is inhibited at least to some degree by the presence of the immunosuppressive agent cyclosporin A (CsA). The molecular identification of the PTP is still

controversial and may consist of proteins associated with the adenine nucleotide translocase and/or the phosphate/hydroxyl ion exchanger. There is a general consensus, however, that cyclophilin D is necessary for full PTP activation and is the target of inhibition by CsA. The MPT is an attractive hypothesis as a primary mechanism underlying acute neural injury because of its activation by factors known to be associated with cerebral ischemia and because of its sensitivity to inhibition by CsA and other drugs demonstrated to be neuroprotective in some ischemic brain injury paradigms. Although CsA and other drugs that either interfere with or compensate for mitochondrial energy failure may represent strategically sound attempts at inhibiting necrotic cell death caused by ischemia and reperfusion, recognition that apoptotic molecular pathways also contribute

to ischemic neurodegeneration has led to alternative mitochondria-targeted antiapoptotic interventions.

4. MITOCHONDRIAL MECHANISMS OF APOPTOTIC DEATH AFTER CEREBRAL ISCHEMIA

4.1. Mitochondrial apoptotic pathways

Progress in the study of programmed cell death and apoptosis over the last two decades led to the unexpected discovery that in addition to their bioenergetic and metabolic roles, mitochondria are also the central regulators of the intrinsic (mitochondrial) apoptotic pathway. In some cells (classified as type II cells), including neurons, mitochondria also play a major role in the extrinsic (DR) apoptotic pathway that is induced by DR ligands such as tumor necrosis factor alpha or Fas ligand.

The current understanding of mitochondrial involvement in apoptosis indicates that the key event in this process is the permeabilization of the outer mitochondrial membrane (OMM) and release of apoptogenic proteins from the mitochondrial intermembrane space into the cytosol, leading to cell demise in a caspase-dependent or -independent manner. The best characterized apoptogenic proteins include cytochrome c (Cyt C), AIF, Smac/DIABLO, endonuclease G, and HtrA2/Omi. The strategic positioning of mitochondria at the intersection of cell death and survival pathways is highlighted by the dual role (with respect to cell death and survival), of many apoptogenic proteins. In addition to the classic example of Cyt C, other apoptogenic proteins initially identified as death inducers when released into the cytosol (e.g., AIF and HtrA2/Omi) were also found to promote neuronal survival and resistance to oxidative stress at their mitochondrial location.

The release of apoptogenic proteins from mitochondria can occur through two distinct mechanisms involving either selective OMM permeabilization by protein/proteo-lipidic pores or nonspecific (mechanical) rupture of the OMM. The most important regulators of the intrinsic pathway of apoptosis are the Bcl-2 family proteins that regulate primarily OMM permeability, although other extra-mitochondrial mechanisms (e.g., endoplasmic reticulum related) are also described. Selective permeabilization of the OMM is triggered by the BH3-only subgroup of Bcl-2 proteins (e.g., Bid, Bim, Bad, Noxa, Puma). Specific stimuli or non-specific cell stress can induce activation of constitutively expressed BH3-only proteins (BOP) (e.g., Bid, Bim, Bad) and/or trigger the expression of several additional BH3 only-proteins (e.g., Noxa, Puma), all of which translocate to mitochondria. The activity of BH3-only proteins at

mitochondria results in oligomerization of multidomain pro-apoptotic Bcl-2 proteins (e.g., Bax/Bak) and permeabilization of the OMM through pore formation. This selective OMM permeabilization occurs without loss of the inner mitochondrial membrane (IMM) integrity and is antagonized by Bcl-2-like antiapoptotic members (e.g. Bcl-2, Bcl-x_L, Bcl-w, and Mcl-1).

Another mechanism of release of apoptogenic proteins involves nonselective rupture of the OMM. Most commonly this occurs after PTP opening, osmotic swelling of mitochondria, and physical rupture of the OMM. Although hallmarks of MPT (i.e., loss of IMM potential [$\Delta\Psi$], swelling of mitochondria, and protection by CsA) are observed in some cases in dying cells that display classic apoptotic morphology, the role of MPT in apoptosis has been controversial. MPT was proposed initially as a universal mechanism of mitochondrial apoptotic death accounting for the release of apoptogenic proteins. Recent studies using cells from cyclophilin D-deficient mice demonstrate that MPT is not required for apoptosis. In contrast, cyclophilin D deficiency protects cells against oxidative stress-induced necrotic death. Regardless of its requirement for apoptosis, the importance of the MPT mechanism in pathological neuronal death is highlighted by recent findings that animals deficient in cyclophilin D display a marked resistance to ischemic brain injury.

The involvement of mitochondria-dependent death pathways in brain ischemia has been demonstrated in both focal and global ischemia using small and large animal models, and evidence for their activation has also been found humans. Although both pathways appear to play critical roles in ischemia/reperfusion-induced neuronal death, their relative contribution varies greatly depending on the model (focal/global), brain region (cortex vs. hippocampus), age (immature vs. mature), and gender. Such results highlight the need for a better understanding at a molecular level and improved recognition in a clinical context of potentially distinct phenotypes of ischemic injury that are age- or gender-dependent. Similarly, the data support further development of targeted therapies for ischemic injury (i.e., specific for the immature vs. adult brain and gender-specific). Critical regulators of the mitochondrial-apoptotic pathway in ischemic brain injury are discussed next.

4.2. Bcl-2 family proteins

Bcl-2 family proteins regulate cell death pathways by acting at the mitochondrial level where they control the release of death-inducing proteins from the

Table 14-1. Bcl-2 family proteins in brain ischemia/reperfusion

Protein		Effect	Model/cells
Bcl-2	Over-expression	Decreased infarct size	MCAO
Bcl-X _L	Over-expression/ protein transduction	Decreased infarct size	MCAO
Bcl-w	Over-expression	Decreased infarct size	MCAO
Bax	Deletion	Decreased infarct size	Neonatal HI
Bad	Deletion	Decreased infarct size	Neonatal HI
Bid	Deletion	Decreased infarct size	MCAO
	Deletion	No effect	Neonatal HI
Bim	Deletion	Decreased infarct size	Neonatal HI
Puma	Deletion	No effect (infarct size, neurological deficit)	MCAO
Noxa	Antisense	Decreased infarct size	MCAO

HI, hypoxia-ischemia; MCAO, middle cerebral artery occlusion.

mitochondrial intermembrane space to the cytosol through regulation of the OMM permeability. Recent studies indicate that Bcl-2 family proteins also affect this process indirectly through their actions at other intracellular locations (i.e., at the endoplasmic reticulum level) by regulating Ca²⁺ fluxes. Numerous studies document a role of for both pro- and antiapoptotic Bcl-2 family proteins in neuronal injury and survival in pathological conditions such as ischemia.

The Bcl-2-like proteins protect neurons and other cell types against a wide variety of apoptotic insults. Although labeled as antiapoptotic, these proteins (e.g., Bcl-2, Bcl-X_L) can also protect against necrotic cell death. At least for Bcl-2, a role in the regulation of cell death associated with autophagy (also termed *autophagic cell death*), through its interaction with the autophagy regulator protein beclin-1, is also documented. This type of cell death, characterized morphologically by the lack of chromatin condensation and presence of massive autophagic vacuolization of the cytoplasm, has been also shown to contribute to cell death after ischemic brain injury. Early studies indicated that overexpression of Bcl-2 exerts a powerful neuroprotective activity in cerebral ischemia, as well as in other models of acute or chronic brain injury. Subsequent studies reported similar neuroprotective properties against ischemic injury for several other antiapoptotic Bcl-2 proteins, including Bcl-X_L, Mcl-1, and Bcl-w (Table 14-1). The strong neuroprotective effect of Bcl-2 likely results from its multiple prosurvival activities and is especially important in brain ischemia in which multiple neuronal death types (apoptotic, necrotic, and autophagic) are frequently observed.

Although not completely understood, the antiapoptotic activity of Bcl-2 and related proteins is in part due to their ability to heterodimerize with Bax/Bak-type (multidomain) proapoptotic proteins and/or to sequester BH3-only proteins. According to the “rheostat” model proposed by the late Stanley Korsmeyer, Bcl-2-like antiapoptotic proteins bind and neutralize proapoptotic Bcl-2 proteins, and their relative balance determines cell death or survival. BH3-only proteins display distinct binding affinities for individual Bcl-2-like proteins, and their killing efficiency correlates with their ability to target and inactivate multiple prosurvival proteins.

In addition to the Bax/Bak neutralizing activity, the antiapoptotic and antinecrotic activity of Bcl-2-like proteins involves regulation of other mitochondrial processes, including mitochondrial Ca²⁺ uptake capacity, MPT, and redox state. Studies on Bcl-2, Bcl-X_L, and Mcl-1 indicate that their cytoprotective activities are in part due to increased protection against oxidative stress via elevating the expression of enzymes actively involved in the defense against ROS. This effect may involve modulation of transcription factor activity and subsequent expression of antioxidant enzymes. It now appears that upregulation of the antioxidant defense systems by Bcl-2 is a preconditioning response to the elevation of mitochondrial ROS production by increased Bcl-2 expression. This response may explain Bcl-2 protection against death caused by oxidative stress throughout the cell, including at the mitochondrial level, where Bcl-2 protects against Ca²⁺ and peroxide-induced MPT activation. This antioxidant component of the anti-death Bcl-2 proteins may be particularly important in acute ischemic injury in which excitotoxicity and oxidative stress play major roles in neuronal cell death.

The role of the multidomain proapoptotic protein Bax has been extensively studied, and there is now strong evidence for involvement of Bax in neuronal death after cerebral ischemia both in the adult and in the immature brain. The mechanism of Bax activation has been the subject of intense study in recent years. Overall, Bax appears as a “molecule on the edge” that potentially can be activated by a wide array of stimuli, either through a specific/instructive pathway involving distinct molecular interactions at multiple levels or through other less specific pathways involving various physicochemical changes of Bax. Both pathways involve changes in the inactive monomeric Bax conformation (similar to an “unfolding” process) and generation of active Bax molecules (aBax; i.e., with an exposed N- or C-terminal domain). Once generated, aBax molecules are either bound and blocked by Bcl-2-like proteins (in surviving cells) or, if accumulated in excess of antiapoptotic

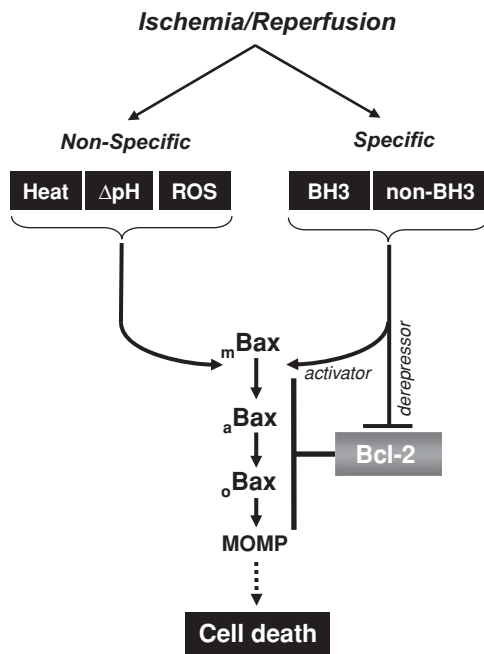


Figure 14-3. Potential mechanisms of Bax activation. The multidomain proapoptotic Bcl-2 family member Bax can be activated through multiple mechanisms that involve either nonspecific physico-chemical modifications of the inactive monomeric Bax molecule (mBax) or specific interactions in a BH3-domain-dependent or -independent manner, with one or more proteins. The best characterized mechanism of Bax activation involves the activity of BH3-only proteins (BOP). Direct activator BOPs (e.g., Bim, Bid) bind Bax directly and promote its conformational change. Derepressor/sensitizer BOPs (i.e., Bad, Noxa) promote activation of Bax indirectly through binding to Bcl-2-like antiapoptotic proteins and release of “pre-activated” Bax molecules sequestered by these proteins. Although some BOP can act both as activators and de-repressors (e.g., Bim, Bid, and, potentially, Puma), others are thought to act exclusively as de-repressors (e.g., Bad, Noxa). Similar to BOP, some non-Bcl-2 family proteins can also bind Bax (direct activation mechanism) or Bcl-2 like proteins (de-repressor mechanism). Nonspecific mechanisms of Bax activation include heat, changes in intracellular pH, and oxidative changes of Bax molecules induced by increased ROS generation. Activated Bax (aBax) translocates to mitochondria, where it is either bound and neutralized by antiapoptotic Bcl-2-like proteins or, if present in excess, assembled into Bax oligomers (oBax). Bax oligomers form pores in the OMM, leading to its permeabilization (MOMP) and release of apoptogenic proteins.

binding partners, assembled as Bax oligomers (oBax) that cause pore formation in the OMM (Figure 14-3).

The multistep process of Bax-dependent pore formation can be modulated at several levels by numerous factors, including proteins that “sequester” Bax in the cytosol (i.e., Humanin, Ku70, 14-3-3), the lipidic composition of the OMM, and so forth. The specific/instructive pathway of Bax activation is highly regulated at multiple levels. Its induction most commonly involves activation of the BH3-only subgroup of Bcl-2 proteins, but can also occur in response to other non-Bcl-2 family proteins (e.g., p53). These proteins can be viewed as inverse

chaperones in that they assist the unfolding (rather than folding) of Bax into activate conformations. In addition, nonspecific pathways of Bax activation have also been described, resulting from structural modifications of mBax induced by heat, pH changes, or oxidative stress. This process can also be easily replicated in vitro (e.g., by detergents). It is not known whether all of these mechanisms, particularly the nonspecific ones, are involved in Bax activation after ischemic brain injury. The efficacy of hypothermia in reducing ischemic brain injury may suggest a role for such nonspecific mechanisms, not only for Bax, but also for activation of many other mediators of neuronal dysfunction and death, including the MPT. If this is the case, therapeutic interventions focusing exclusively on the specific molecular pathways of activation are not likely to provide full (or persistent) protection against cell death. Although both nonspecific and specific pathways of Bax activation may lead to a “common” effector molecule/conformation (i.e., oligomeric oBax), the intermediate steps may be quite distinct. Therefore, it is not clear whether a single small-molecular inhibitor such as those developed recently will efficiently block Bax activation through multiple pathways. Combinatorial therapies of ischemic brain injury should therefore continue to investigate concomitant targeting of both nonspecific and specific molecular alterations.

The BH3-only proteins act upstream of multidomain proteins as sensors/transducers of apoptotic stimuli and trigger the activation of the multidomain Bax/Bak proteins. Despite employing similar mechanisms of action, the contribution of individual BH3-only proteins to apoptosis in different models of brain injury is at least partially selective. For instance, Bid deficiency protects neurons against ischemic injury in the adult brain, Dp5/Hrk deficiency protects against axotomy-induced neuronal death, and Bim and Bad contribute to seizure-induced neuronal death. Multiple BH3-only proteins—including Bad, Bid, Bim, Puma, Noxa, Dp5/Hrk, and Bnip3—are upregulated and activated and subsequently translocate to mitochondria after cerebral ischemia. Experiments using either knockout mice (e.g., Bid, Bad, Bim) or antisense down regulation (e.g., Noxa) indicate that although some BH3-only proteins are required for initiation of neuronal death after cerebral ischemia, activation of other BH3-only proteins (e.g., Puma) is dispensable (Table 14-1). Concomitant upregulation and activation of multiple BH3-only proteins in response to cerebral ischemia reflects the functional redundancy among these proteins, thereby weakening their usefulness as individual targets for pharmacological intervention. This redundancy is also evident during brain development because no major defects in apoptotic death

within the brain are observed in mice lacking the expression of individual BH3-only proteins (e.g., Bid, Bad, Bim).

Recent studies revealed that the involvement of many Bcl-2 family proteins in brain cell death and survival is multifaceted and much more subtle than previously appreciated. Some of these proteins are involved in part of the extensive cross-communication between cell death/survival pathways previously considered distinct (e.g., regulation of apoptotic, autophagic, and necrotic death by Bcl-2; Figure 14-1). Similarly, several Bcl-2 family proteins, initially identified and studied mostly as cell death/survival mediators, are now known to function as regulators of basic physiologic processes in healthy cells (e.g., Bad involvement in glucose homeostasis and Bcl-x_L in mitochondrial morphology and synaptic neurotransmission). Successful targeting of Bcl-2 proteins for therapeutic purpose is also complicated by the fact that many Bcl-2 family members are expressed as multiple isoforms, often with opposed functions. In some cases, isoforms are species-specific. For instance, two isoforms of Mcl-1 are expressed in humans and only one in other mammals. Species selective isoform diversity is one of several factors that limit rapid translation of experimental findings from animal models to human pathology.

Despite the complexity of pathogenic mechanisms of ischemic brain injury, remarkable progress in basic science and understanding of cell death mechanisms over the last two decades led to development of several small-molecule drugs and therapeutic peptides/proteins (Table 14-2), some of which are already in clinical trials for various neurodegenerative diseases or cancers. Among these, caspase inhibitors protect neurons against cell death in animal models of brain ischemia. However, the potential switch from apoptosis to necrosis in the presence of caspase inhibition, as well as recognition that caspase-independent pathways are also activated in the postischemic neurons (i.e., AIF release from mitochondria), suggest that interfering with upstream steps in the death cascade should provide increased protection.

The discovery of the essential role played by the multidomain proapoptotic proteins (Bax and Bak) in activation of the mitochondrial apoptotic pathway and of several endogenous regulators of Bax activation led to the development of several new cytoprotective agents. Among these are a cell-permeable Bax-inhibiting peptide (BIP) derived from the Bax-binding region of Ku70 and a small Bax-sequestering peptide humanin, which, although endogenous, can also be artificially delivered inside cells by attaching it to small cell-penetrating

Table 14-2. Mitochondria-related drugs and proteins

Small-molecular inhibitors	Target	Drugs/proteins
	MPT/CypD	Cyclosporin A Methylvaline-4-CsA
	RIP1/MPT	Necrostatin
	Bax inhibition	Dibucaine, propranolol TUDCA Humanin BIP Bcl1, Bcl2
	Caspase inhibition	zVAD
	PARP inhibition	3-AB
	p53 inhibition	PFT- α
Protein transduction	Peptides	TAT-NBD TAT-Bcl-2 BH4 peptide TAT-Bcl-x _L BH4 peptide
	Proteins	TAT-Bcl-x _L /TAT-FNK TAT-GDNF

GDNF, glial cell line-derived neurotrophic factor; TUDCA, tauroursodeoxycholic acid.

peptides (i.e., the HIV-1 transactivator of transcription [TAT] protein transduction domain [PTD]). Tauroursodeoxycholic acid inhibits Bax translocation to mitochondria and has been shown to be neuroprotective in a rat stroke model. Two small-molecule inhibitors of Bax channel activity (Bcl1 and Bcl2) that inhibit Cyt C release from mitochondria have been recently discovered. Inhibition of Bax channel activity by Bcl1 and 2 protects against apoptosis and is neuroprotective in an animal model of global ischemia. In addition to pharmacological inhibitors targeting the core apoptotic regulator Bax, p53 inhibitors (e.g. pifithrin- α), PARP-1 inhibitors (e.g., 3-AB) and RIP1-kinase inhibitors (e.g., necrostatin) also confer protection against ischemic brain injury and have therapeutic potential.

The recent development of protein transduction technology has provided a new approach for neuroprotection by facilitating the delivery of proteins and peptides into cells and tissues, including the brain. Although the mechanism by which small cell-penetrating peptides (PTDs) mediate intracellular delivery of various attached cargoes (i.e., peptides or proteins) remains debated, the neuroprotective potential of this strategy has been demonstrated by several studies in cells and models of brain injury in mice. Delivery of the antiapoptotic Bcl-x_L, (e.g., TAT-FNK, a modified Bcl-x_L) or glial cell line-derived neurotrophic factor as fusion proteins with the HIV-1 TAT PTD have been reported to reduce the severity of ischemic injury in mouse models.

4.3. Caspase-dependent apoptosis

The release of Cyt C into the cytosol initiates a molecular cascade leading to caspase-dependent cell death. In the presence of deoxy-ATP (dATP), Cyt C binds to Apaf-1 and triggers activation of the initiator pro-caspase-9 within the apoptosome. Active caspase-9 then activates effector caspase-3 and -7, which in turn cleave a large number of substrates responsible for DNA fragmentation and cell disassembly.

Numerous studies document the contribution of this pathway to neuronal death in both focal and global cerebral ischemia models. In models of focal cerebral ischemia, the release and relocation of Cyt C from mitochondria to cytosol and the presence of activated caspase-3 is detected in the ischemic penumbra. In addition, caspase-3 deficiency has been shown to render mice partially resistant to ischemic injury. Although most studies indicate that caspase activation occurs in a delayed manner (days or even weeks after the initial injury), activation of both proapoptotic Bcl-2 family proteins (e.g., Bid) and caspase cleavage (caspase-3, -8, -10, -14), possibly mediated by calpains, can also occur as early as 30 minutes during reperfusion after global cerebral ischemia. Some studies indicate that neurons containing activated caspase-3 are additionally present in the necrotic core after focal cerebral ischemia. Although information in humans is much more limited, reports indicate an increase in pro-caspase-3 within hours after ischemic stroke. Similarly, activated caspase-3 and cleavage of PARP have been reported in some neurons several days after cardiac arrest and reperfusion.

Cleavage of PARP-1, a widely used marker of caspase-3 activation, illustrates some of the cross-communication occurring between different cell death pathways (Figure 14-1). Although sustained overactivation of PARP-1 can lead to a necrotic-like cell death, cleavage of PARP-1 by activated caspase-3 can shift the cell death outcome toward apoptosis. Activation of caspase-3 and -9 is endogenously controlled by the inhibitor of apoptosis (IAP) family proteins. Their important role is highlighted by studies indicating that over-expression of the IAP protein X-linked IAP promotes neuronal survival after cerebral ischemia.

4.4. Caspase-independent apoptosis

Classically, apoptosis execution was thought to require activation of the caspase family of cysteine proteases. However, cell death with morphological features of apoptosis, or so-called caspase-independent apoptosis, is now known to contribute significantly to ischemic

brain injury. Although like other forms of cell death, caspase-independent apoptosis cannot be defined by a single linear biochemical cascade, key players have emerged. Not surprisingly, attention has once again focused on mitochondria, as well as a second family of destructive cysteine proteases, the calcium-activated calpains.

The first evidence for the existence of a caspase-independent mitochondrial apoptosis factor came from the treatment of purified nuclear extracts with a protein mixture derived from calcium-treated mitochondria (Figure 14-4). The mitochondrial protein responsible for nuclear fragmentation, dubbed AIF for “apoptosis-inducing factor,” is now known to translocate from the mitochondrial intermembrane space to the nucleus after OMM permeabilization. AIF nuclear localization and its ability to fragment DNA require association with cyclophilin A, a cytosolic peptidyl prolyl cis-trans isomerase (distinct from cyclophilin D) that co-translocates to the nucleus. Mice with an attenuation in either AIF or cyclophilin A expression exhibit neuronal sparing after the induction of experimental ischemic brain injury. The engineering of an AIF mutant containing a nuclear export sequence elegantly confirmed the importance of nuclear translocation in AIF-mediated apoptosis.

Nuclear PARP-1 was identified as an upstream mediator of AIF-dependent cell death. This enzyme catalyzes the formation of poly (ADP)ribose (PAR) and nicotinamide from NAD⁺ and functions in DNA repair. However, when DNA damage becomes excessive (e.g., oxidative damage after ischemia/reperfusion brain injury), overactivation of PARP and resulting NAD⁺ depletion can occur. The brains of PARP-knockout male mice are remarkably spared from ischemic injury induced by transient middle cerebral artery occlusion. The delivery of neutralizing AIF antibodies to cultured neurons attenuates PARP-dependent death induced by either activation of calcium-permeable N-methyl-D-aspartic acid-type glutamate receptors or by the DNA alkylating PARP activator N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). The mechanisms by which PARP initiates a caspase-independent program of cell execution remain to be fully elucidated but likely involve both metabolic dysfunction resulting from cytosolic and perhaps mitochondrial NAD⁺ catabolism as well as the production of toxic PAR polymers. Interestingly, in contrast to PARP-1 knockout males, PARP-1 knockout female mice exhibit increased rather than decreased infarct volume after focal ischemia/reperfusion. Sex-based differences in biochemical death pathways, although poorly studied, are now receiving increasing attention. Preliminary data indicate that sex-based differences in the

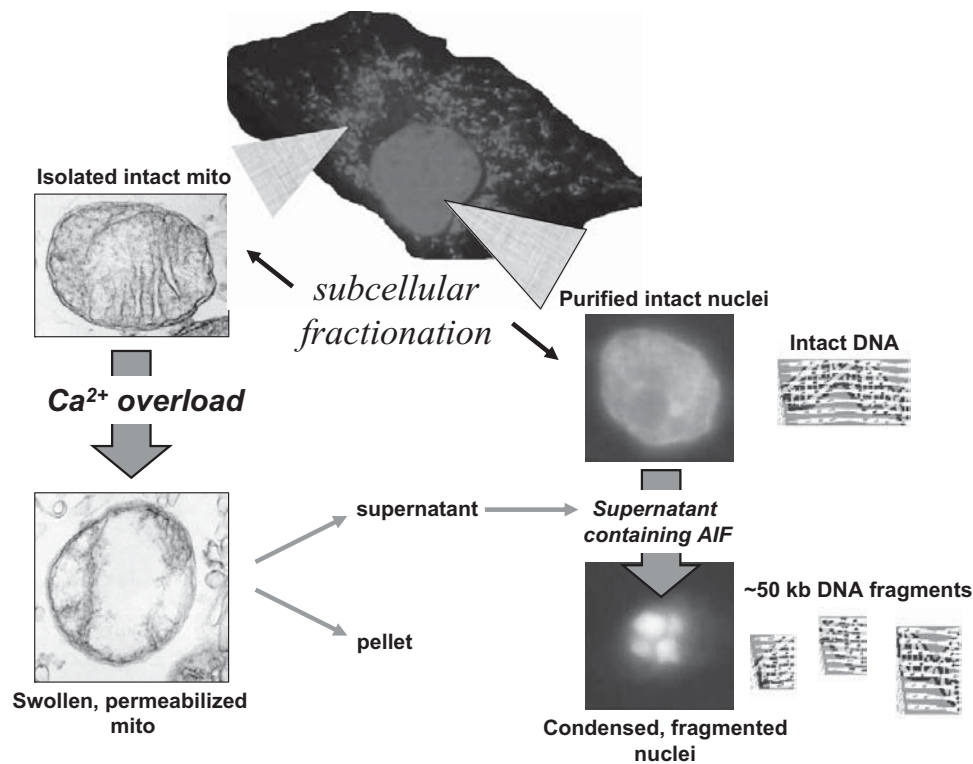


Figure 14-4. Identification of a caspase-independent mitochondrial apoptosis-inducing factor using an in vitro reconstitution assay with subcellular fractions. Depicted is a cell displaying mitochondrial AIF immunostaining and Hoechst nuclear counterstaining. AIF was first identified by (1) treating purified mitochondria with calcium, (2) isolating proteins released into the solution, (3) adding the protein mixture to extracted nuclei to induce nuclear fragmentation, and (4) subfractionating the protein mixture to identify the active component. See Color Plate 14.

sensitivity of cells and animals to injury are far more widespread than previously suspected. Although some of the differences are due to the action of circulating sex hormones (e.g., estrogen and progesterone), intrinsic genetic-based differences in cultures of XX versus XY cells are also observed.

4.5. Calpains in ischemic neural cell death

Until recently, the steps leading to the nuclear translocation of AIF in caspase-independent neural apoptosis were not understood. It is now known that AIF release from mitochondria requires multiple steps. Outer membrane permeabilization is an initial requirement that occurs downstream of Bid-induced Bax insertion into the OMM or after swelling-induced membrane rupture associated with opening of the Ca²⁺-activated PTP. A second requirement is proteolysis of the membrane-embedded N-terminus of AIF that mediates its detachment from the inner membrane. This processing is performed by calpain-1 endogenous to the mitochondrial intermembrane space as well as by extramitochondrial calpain-1, which accumulates at the mitochondria

after oxygen/glucose deprivation. Calpain-cleaved Bid releases AIF from isolated brain mitochondria, but only in the presence of active exogenous calpain-1 that permeates the outer membrane and cleaves AIF. Although the consequences of OMM permeabilization have normally been associated with the release of apoptogenic proteins, this experiment established that the passage of large proteins across the outer membrane after Bid/Bax-mediated permeabilization is bidirectional. The additional finding that calpain-cleaved but not full-length Bid releases AIF at similar concentrations demonstrates that calpain also augments Bid-induced outer membrane permeabilization.

MNNG treatment of mouse embryonic fibroblasts derived from knockout mice is a convenient way to genetically dissect additional biochemical pathways involved in AIF and PARP-dependent cell death. Calpain knockout fibroblasts are resistant to MNNG-induced apoptosis. However, caspase-3, caspase-9, cathepsin B, or cathepsin L ablation has no effect on this form of injury. These findings demonstrate that PARP-dependent cell death is calpain-dependent but caspase-independent and does not require the cysteine class

of lysosomal proteases. Mitochondrial AIF release does not occur in Bax knockout or calpain knockout cells in the MNNG model, confirming the requirement for both outer membrane permeabilization and proteolysis for AIF efflux. In keeping with these genetic findings, pharmacological inhibition of Bid or over-expression of the calpain inhibitor calpastatin blocks the release of AIF after focal or global ischemia, respectively, affording significant neuroprotection in vivo. Knockdown of AIF via the naturally occurring harlequin (Hq) mutation or siRNA delivery confirms that the AIF-mediated death pathway contributes to the size of the infarct after transient focal or global ischemia. Significantly, viral delivery of wild-type AIF but not a calpain-resistant mutant restores the sensitivity of AIF-depleted hippocampal CA1 neurons to injury after transient global ischemia. Collectively, these experiments confirm that calpain processing of AIF plays a crucial role in caspase-independent cell death in vivo.

AIF is by no means the only target of calcium-dependent calpain proteases. Over-expression of the calpain inhibitor calpastatin confers additional protection to AIF-depleted neurons subjected to oxygen/glucose deprivation, demonstrating that the detrimental effects of increased calpain activity are clearly mediated by the processing of multiple targets. Several proteins that participate in classical, caspase-dependent apoptosis are also calpain substrates, including caspases-3, -7, -8, -9, and -12 as well as Bcl-2 family members Bid, Bax, and Bcl-x_L. Cleavage of Bcl-2 family members by calpain favors both caspase-dependent and caspase-independent apoptosis by releasing mitochondrial cytochrome c, Smac/DIABLO, and AIF. However, proteolytic inactivation of caspases after calpain over-activation favors caspase-independent apoptotic or necrotic cell death. Intriguingly, the processing of Bcl-x_L by calpain (or caspase) serves the dual purpose of inactivating a potent antiapoptotic molecule and generating a pro-death C-terminal fragment that has been detected in the postischemic hippocampus in association with increased mitochondrial membrane conductance. Because most Bcl-2 family death regulators are expressed at higher levels in the developing brain compared with the adult, the immature brain may be especially vulnerable to the induction of apoptotic pathways associated with cleavage-dependent Bcl-2 family regulation after ischemic injury.

The extent of energy impairment, intracellular calcium deregulation, and oxidative stress all regulate whether cells die by caspase-dependent apoptosis, caspase-independent apoptosis, or acute necrosis. Caspase-dependent apoptosis requires sufficient ATP

levels for apoptosome activation. Metabolic impairment resulting in the failure of ATP-dependent ion pumps leads to intracellular calcium rises that, if sustained, become irreversible. Cell swelling and a necrotic death characterized by loss of plasma membrane integrity is the frequent result. Caspase-independent apoptosis likely resides in the center of the apoptosis–necrosis spectrum. Large intracellular calcium rises that result from the opening of calcium-permeable glutamate receptors and other cation channels favor calpain protease activation. In addition to potentiating the death-inducing activity of Bcl-2 family proteins and triggering AIF release, calpain-directed proteolysis of caspases inhibits classical apoptosis, leading to caspase-independent cell death. Caspase-independent apoptosis shares many of the morphological features of classical caspase-dependent apoptosis and likely serves as a secondary mechanism of limiting the inflammation response when intracellular ATP depletion impairs the initial programmed cell death response. The roles for calpain proteases in apoptosis after ischemic injury are summarized in [Figure 14-5](#).

5. SUMMARY

Before the seminal observation that cytochrome c is both released from mitochondria and stimulates caspase activation during many apoptotic cell death paradigms, it was believed that metabolic failure was the primary role of mitochondria in neural cell death after cerebral ischemia and reperfusion. Despite the dramatic increase in our knowledge of pathological apoptosis, mitochondrial bioenergetic dysfunction is still considered a major cause of neuronal death after cerebral ischemia and must be ameliorated for clinical outcome to be improved. Opening of the inner membrane permeability transition pore in response to abnormal mitochondrial Ca²⁺ accumulation and oxidative stress is widely considered to be at least one important mechanism of metabolic failure during acute brain injury. Moreover, pharmacological inhibitors of the mitochondrial permeability transition are currently being tested in clinical trials for acute brain injury. Other mechanisms include direct inactivation by reactive O₂ and nitrogen species of critical mitochondrial metabolic enzymes in the tricarboxylic acid cycle and the electron transport chain. Catabolism of both cytosolic and mitochondrial NAD(H) by PARP-1 in response to its activation by oxidative stress is another important cause of metabolic dysfunction. Drugs or other interventions that reduce oxidative stress and inhibit PARP-1 activity also show promise as neuroprotectants.

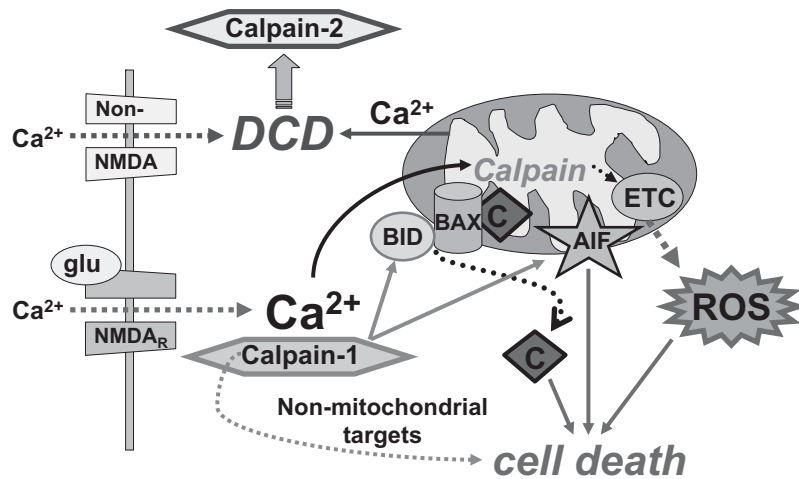


Figure 14-5. Calpain pathways contributing to ischemic brain injury. Calpain-1 activation after ischemic injury is linked to Ca^{2+} influx through NMDA receptors. Proteolysis of Bcl-2 family members and AIF contribute to the pathological release of cytochrome c (C) and truncated AIF, respiratory inhibition, and elevated ROS production. Mitochondrial sequestration of Ca^{2+} may also lead to matrix calpain activation and degradation of electron transport chain subunits, although this remains an active area of investigation. Ca^{2+} entry through non-NMDA receptor channels, mitochondrial calcium release, and failed Ca^{2+} extrusion ultimately lead to catastrophic delayed Ca^{2+} deregulation (DCD), calpain-2 activation, and disintegration of the cell.

Before the point of irreversible cellular metabolic failure, when both the plasma membrane and sub-cellular membranes lose their ability to retain both small and large molecules, apoptotic molecular pathways are typically activated in parallel with the macromolecular degradation that potentially leads to necrosis. These apoptotic pathways can be categorized as either not requiring mitochondrial involvement (extrinsic pathway) or dependent on mitochondria (intrinsic pathway). The intrinsic pathway is either caspase-dependent or -independent on the basis of whether or not cytochrome c-dependent formation of the apoptosome occurs. The intrinsic pathway is activated within 30 minutes in some models of cerebral ischemia and consists of post-translational modification of many proteins and complex protein-protein interactions between both pro- and antiapoptotic members. Pharmacological inhibitors of Bcl-2 are being tested clinically for promoting the death of cancer cells, so it is likely that drugs that inhibit OMM pore formation (e.g., Bax inhibitors) will eventually be tested for cytoprotection, including for that after cerebral ischemia. Calpains, which catalyze Ca^{2+} -dependent proteolysis of both apoptotic and nonapoptotic proteins, are another important potential target of intervention

for ischemic brain injury because they contribute to both apoptotic and necrotic cell death. Hopefully, in time, the clinical outcome after stroke, global cerebral ischemia, and other forms of acute brain injury will improve as effective combined therapies are developed that both preserve cerebral energy metabolism and inhibit the key molecular events that occur at mitochondria, where different apoptotic pathways converge.

ACKNOWLEDGMENTS

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Cell Death in Spinal Cord Injury – An Evolving Taxonomy with Therapeutic Promise

Rajiv R. Ratan and Moses V. Chao

1. INTRODUCTION

The Edwin Smith Papyrus, the only surviving copy of the ancient Egyptian textbook on trauma surgery, shows that the therapeutic challenge of protecting the nervous system after spinal cord injury (SCI) has burdened man for millenia (Haas, 1999). During the past 5,000 years, the sense of urgency surrounding treatment for this important malady has only grown as preventive measures have failed to eradicate it (Gupta et al., 2009; Gupta et al., 2008). Given the daunting challenges in repairing the injured spinal cord according to the accurate anatomic descriptions of the ancient Papyrus, it is not surprising that attention has focused on preventing cell death after injury (Faden & Stoica 2007). This chapter traces some of the intellectual antecedents underlying our current models of death and survival in the spinal cord after trauma and culminates in a discussion of the potential therapeutic implications of the field's journey to date, focusing on the concept of apoptosis and its now appreciated variants.

2. HISTORICAL ANTECEDENTS

An important turning point in our understanding of the spinal cord (and, by extension, spinal cord injury) came from detailed studies of cell death during nervous system development by Rita Levi-Montalcini and Viktor Hamburger in the 1940s and early 1950s (Cowan, 2001; Hamburger, 1992). By performing careful anatomical studies of motor neurons and dorsal root ganglia in chick embryos they showed, somewhat surprisingly, that nervous system development is associated with massive neuronal death. They hypothesized that extensive programmed cell death (up to 80% in some cell populations) during development represents a genetically efficient

way in which to match an overabundant pool of neuronal cell bodies and associated axons with their remote synaptic targets. More specifically, they suggested that neurons (e.g., motor neurons) are deployed via their axons to many targets, but only those few that reach a target with the appropriate trophic factor support will survive (those that do not suffer the less noble consequence of death). The model has proven to be substantially correct, and from these enormously insightful studies arose whole new fields of research on trophic factors and neuronal survival. However, what they failed to do was stimulate widespread interest in programmed cell death as a biological phenomenon with a role that extends beyond the period of development into normal adulthood, aging, or injuries of the brain and spinal cord.

It is in this context that the studies of Kerr and coworkers have had the most impact (Kerr, 2002). They were interested in the histochemical changes that occur in lysosomes after hepatic ischemia. Their model posed that in response to a lack of glucose and oxygen, hepatocytes release their lysosomal contents (proteases) and essentially “digest themselves to death.” To test this hypothesis, the branches going to the left and median lobes of the liver were ligated. As expected, immediately after the occlusion, lysosomal proteases were released into the cell and massive necrosis occurred in areas supplied by terminal hepatic veins. The portal area remained essentially viable, as it was supplied by the hepatic artery. However, during the ensuing days to weeks to months as blood supply and parenchymal mass were aligned, detailed electron micrographic studies revealed cytoplasmic, membrane-bound masses containing normal organelles including intact lysosomes within normal hepatocytes. Kerr et al. inferred that cells were being compartmentalized into small vesicles

containing normal organelles. These small vesicles were then phagocytosed by neighboring hepatocytes, which could be monitored by electron microscopy. This was clearly a manifestation of cell death, and yet it was clearly distinct from necrosis because the plasma membrane stayed intact and there was little inflammatory response. Kerr and coworkers initially called this *shrinkage necrosis*, but the term *necrosis* seemed inappropriate to describe a process that was eerily similar to that described several decades earlier by Viktor Hamburger and Rita Levi-Montalcini in the chick embryo during development (Kerr, 2002). Accordingly, they called it *apoptosis*, derived from the Greek “to fall off,” as in leaves falling from a tree, and in contrast with *mitosis*, in which cells divide and organs grow. The studies of Kerr, Wyllie, and others provided firm experimental grounding to the notion that apoptosis could not only be activated during nervous system development, but like other processes such as division, could also be dysregulated in disease and account for neuronal loss in these contexts (Kerr, 2002).

Ironically, it was not until more than two decades later in the early 1990s that clinical neuroscientists, building on these and more contemporary seminal observations of Horvitz, Croce, Reed, and Korsmeyer on the pro-survival proteins CED-9 in *Caenorhabditis elegans* and Bcl-2 in mammals, began to explore the idea that cell death after injury is not a passive response to random internal destruction. Instead, pathological cell death is a well-orchestrated and deliberate sequence of events that culminates in the ordered dismantling of protein, lipid, and DNA for quiet disposal by professional phagocytes (Graninger et al., 1987; Reed et al., 1987).

Over the past 15 years, the SCI field has been wrestling with a number of questions, which we will attempt to further stimulate in this review:

- (1) After traumatic damage to the spinal cord, is apoptosis the inappropriate result of aberrant signaling or alternatively the appropriate result of irreversible internal damage?
- (2) Even if death is appropriately activated in damaged cells, can we understand enough about how apoptosis triggering damage is initiated in SCI to prevent it? Even more ambitiously, can we learn more about how to repair a cell once it has assessed its level of damage as irreversible to prevent the functional disability in SCI?
- (3) Finally, can cell damage be rescued and apoptosis prevented in SCI in a manner that does not affect the physiologic death of immune cells and precancerous cells inside and outside of the nervous system?

3. CELL DEATH IN THE ACUTE PHASE OF SCI: BEYOND THE APOPTOSIS AND NECROSIS DICHOTOMY

Answers to the challenging questions posed above can only be obtained via a clear understanding of the sequence of events triggered by traumatic injury to the cord. The events after acute spinal cord trauma have been divided into three secondary phases: acute, sub-acute, and late (Springer et al., 1997a, 1997b; Beattie et al., 1997; Buchli et al., 2007). Trauma to the vertebral column induces acute laceration, stretching, and compression of the spinal cord that propagates radially and longitudinally from the impact site. As a result of these primary events, ischemia (insufficient perfusion) and edema (swelling) dominate in the acute phase, and these are mutually reinforcing. Spinal cord perfusion pressure (SCPP) is defined as the difference between the mean arterial pressure (MAP) and the intraspinal pressure (ISP): $SCPP = MAP - ISP$ (Augoustides, 2008; Shi et al., 2007). This represents the pressure gradient driving spinal cord blood flow (SCBF) and hence oxygen and metabolite delivery. Vessels are often sheared as a result of trauma, thus leading to decreased mean arterial pressure and consequent deficiency in metabolite delivery. Traumatic ischemia is further exacerbated because ISP increases as a result of edema, creating a vicious cycle propagating ischemia and damage.

Hypoxia and ischemia lead to aberrant accumulation of glutamate in spinal cord synapses, leading to the now classical phenomenon of excessive activation of cell surface glutamate receptors known as *excitotoxicity* (Taccola et al., 2008) (Figure 15-1). Ischemia-induced injury was once held to uniformly result in necrosis, but beginning with the studies of Kerr and coworkers in the early 1970s, it has become clear that hypoxia and hypoglycemia can activate controlled paths to cell death that are dependent or independent of excitotoxicity and dependent or independent of caspases (Citron et al., 2008). In the cord, the models for understanding excitotoxicity, the neurons it affects, and the precise mechanisms of death are not as well established as those in the cortex, and the field has relied on a firm necrosis–apoptosis dichotomy in which to invoke disease mechanisms (Feng et al., 2008). It has become clear that oxygen-glucose deprivation in the spinal cord can induce forms of cell death that are neither classical apoptosis or necrosis and may include parthanatic death, necroptosis, and autophagy (Kanno et al., 2008). Just over a decade ago, the therapeutic excitement surrounding apoptosis depended largely on the assumption that the morphological features of classical apoptosis implicated one or a few signaling pathways. We now

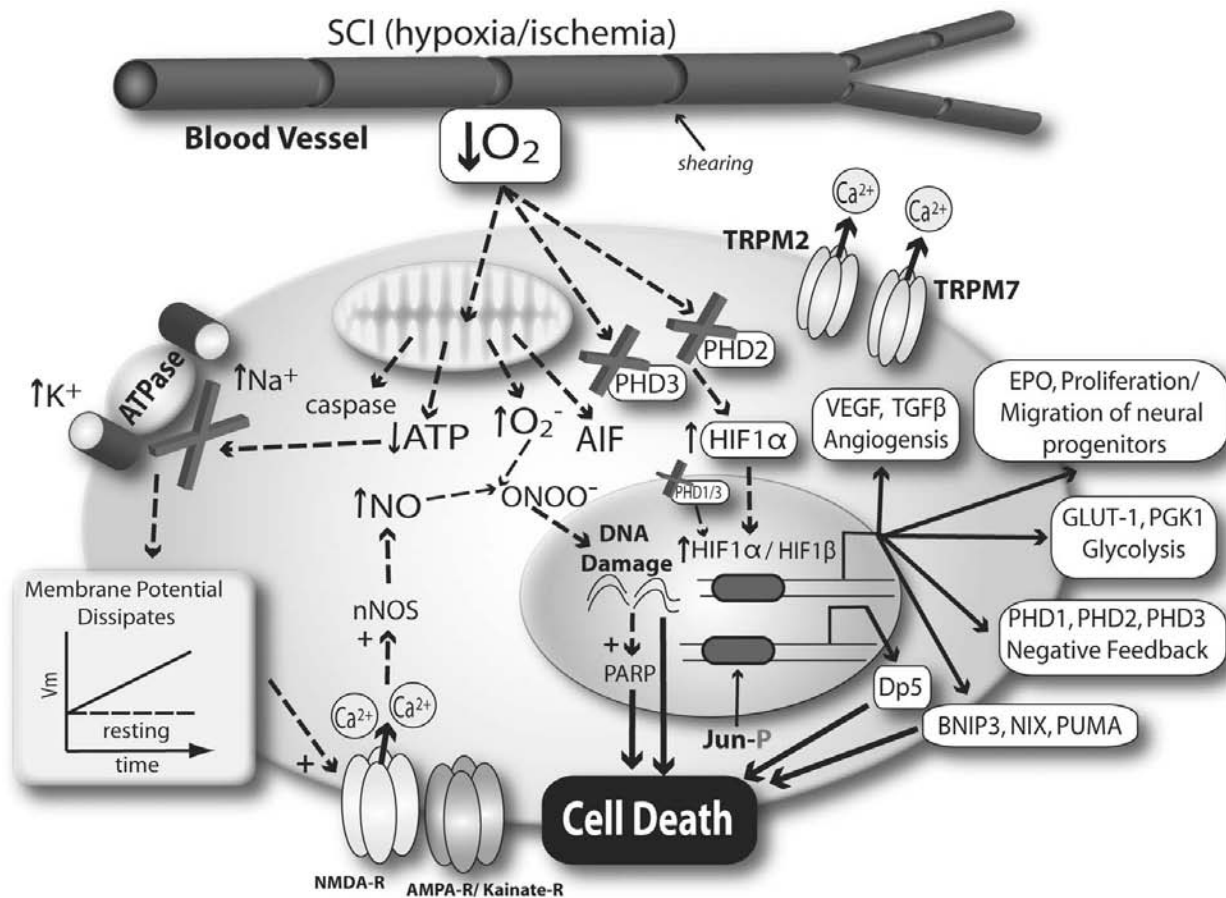


Figure 15-1. Extrinsic and intrinsic signals of cell death and survival after spinal cord injury. Trauma to the cord induces mechanical shearing of axons and blood vessels. Acute shearing of vessels leads to diminished perfusion and cytotoxic edema. Both of these events conspire to reduce the supply of oxygen and glucose below a critical threshold, called ischemia. Energy stores are reduced, leading to aberrant accumulation of the excitatory neurotransmitter glutamate and excessive activation of cell surface receptors. These receptors gate calcium and lead to cytosolic activation of calpain and nNOS among other proteins. Peroxynitrite and calpain lead to PARP activation and further consumption of NAD⁺. PARP activation causes PAR accumulation and is one of the factors leading to release of AIF from the mitochondria to the nucleus. Hypoxia and/or glucose deprivation lead to intrinsic stabilization or activation of pro-death transcriptional factors, including HIF-1 and Jun. These factors bind to the promoters of pro-death proteins, leading to apoptotic, necrotic, or parthanotic forms of cell death. The ability of these transcriptional activators to induce pro-death or prosurvival gene expression is dependent on context and can be modulated by redox state or pH. The complexity of this partial picture of cell death pathways indicates that single, target therapy is unlikely to be successful. EPO, erythropoietin; GLUT-1, glucose transporter 1; PGK1, phosphoglycerate kinase 1; TGF β , transforming growth factor beta; VEGF, vascular endothelial growth factor. See Color Plate 15.

know that and that there are many, if not scores of, effector pathways linking primary injury to controlled forms of cell death (Bredesen, 2007). Excitotoxic cell death in neurons that is not necrosis is caspase-independent and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) negative but requires the translocation of mitochondrial apoptogenic proteins apoptosis-initiating factor (AIF) and endonuclease G (endoG) to the nucleus (Cregan et al., 2004; Yu et al., 2003). Consistent with a similar pathway in motor neurons after traumatic SCI, it was shown that AIF and endoG are found in motor neurons (Yu et al., 2006) (Figure 15-1).

The upstream events required to induce translocation of AIF from the mitochondria are not uniform and have not been studied in detail in the spinal cord. In some excitotoxicity models, gating of calcium via ionotropic glutamate receptors leads to subplasmalemmal activation of calmodulin-dependent neuronal nitric oxide synthase (Soriano et al., 2008). Ambient levels of nitric oxide rise and are free to react at nearly diffusion limited rates with superoxide to form the toxicant peroxynitrite. Prior studies have shown that neuronal nitric oxide synthase (nNOS) is channeled to ionotropic glutamate receptors via a PSD-95 (postsynaptic density protein 95)

scaffold, coupling excessive N-methyl-D-aspartic acid (NMDA) receptor activity to NO-dependent neurotoxicity (Aarts et al., 2002; Sun et al., 2008). Indeed, peptides that disrupt the interaction between PSD-95 and NMDA receptors have been shown to induce durable neuroprotection at supratentorial sites in the CNS. Information is only beginning to emerge on the role of PSD-95/nNOS interaction after SCI. After experimental SCI, PSD-95 was associated with neuronal cell bodies and dendritic synapses in the ventral horn, presumably on motor neurons. PSD-95 is also expressed in oligodendrocytes (Cheng et al., 2008). In all of these cell types using histochemical and biochemical methods, PSD-95 was colocalized with nNOS.

The localization of NMDA receptors with PSD-95 and nNOS suggests that trauma and hypoxia in the cord after SCI may induce peroxynitrite formation via inotropic glutamate receptor activation (Figure 15-1). Consistent with these results, 3-nitrotyrosine staining is seen after spinal cord injury in neurons and oligodendrocytes (Xu et al., 2001), although the pattern of staining depends on the primary injury mechanism – contusion, dislocation, or distraction. In other systems, peroxynitrite is believed to propagate dyshomeostasis of calcium by activating a nonspecific cation channel members known as TRPM (transient receptor potential cation channel subfamily M) channels (Aarts et al., 2003). Once activated, these channels gate calcium, leading to further activation of calpains and nNOS. Calpains are multi-isoform, cysteine proteases that, like caspases, selectively degrade structural and repair proteins to ensure the proper demise of the cell. Several studies using compression or contusion injury have demonstrated that calpain inhibition sustains functional recovery (Sribnick et al., 2007). In addition to activating calcium influx and calpain activation, peroxynitrite can also damage DNA, leading to activation of the DNA repair enzyme, poly (ADP) ribose polymerase (PARP). PARP-1 activation leads to poly ADP ribosylation at the expense of NAD⁺. As NAD⁺ decreases, PAR increases (Genovese & Cuzzocrea, 2008). Recent studies have placed calpain activation downstream of PAR accumulation in cells, although it could be that calpain activation downstream of PARP simply reflects a compromise in energy-dependent calcium homeostasis (Moubarak et al., 2007) (Figure 15-1).

As is clear from the previous discussion, excitotoxicity during the acute phase of spinal cord injury results in the activation of a number of pathways of injury that derive from excessive calcium and peroxynitrite. These events conspire to lead to activation of the selective protease calpain (Ray et al., 2001a, 2001b). Calpain can lead to the processing and activation of a number of targets,

including the apoptogenic proteins Bax and AIF (Figure 15-1), further reinforcing caspase-independent forms of programmed death. The therapeutic implications of these parallel but interacting mechanisms of excitotoxicity in the cord indicate that blocking one type of receptor or one downstream signaling component will likely not provide functional recovery. As expected, this has been the empirical experience of the SCI community. Single or combinatorial approaches must be developed to block the secondary effects of trauma, including aberrant glutamate receptor stimulation in the cord. More recent studies suggest that α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate/Kainate and NMDA receptors combine to mediate neuronal and oligodendroglial loss in the acute phase (Bakiri et al., 2008).

4. INTRINSIC MEDIATORS OF ACUTE CELL DEATH: EXCITOTOXICITY VERSUS HIF OR JUN

The therapeutic challenge rises because, in parallel to these excitotoxic events, cell death pathways may also be activated in the early phase of SCI via other secondary mechanisms besides excitotoxicity. As mentioned previously, shearing of vessels and swelling decreases perfusion and tissue levels of oxygen and glucose. Ischemia leads to a number of adaptive events in the cell, including the stabilization of the transcriptional activator, hypoxia-inducible factor-1 (HIF-1) (Ratan et al., 2007) (Figure 15-1). HIF-1 stability is regulated via the activity of oxygen, 2-oxoglutarate, and iron-dependent dioxygenases known as the HIF-prolyl 4 hydroxylase (HIF-PHDs). These enzymes regulate HIF stability by hydroxylating HIF on evolutionarily conserved proline residues in the oxygen-dependent domain of the protein. Hydroxylation of HIF allows the recruitment of the E3 ubiquitin ligase, Von Hippel Lindau protein, and the consequent ubiquitination and degradation of HIF. Under conditions of ischemia, HIF regulates a number of genes involved in adaptation to hypoxia, including erythropoietin, vascular endothelial growth factor, and glycolytic enzymes. Some of these downstream targets have been shown to be efficacious when added exogenously (e.g., erythropoietin, vascular endothelial growth factor) in contusion injury to the cord (Choi et al., 2007; King et al., 2007; Okutan et al., 2007).

By contrast and further testimony that cell death is part of an adaptive repertoire are the findings that BH3-only pro-death genes such as BNIP3, PUMA, and NOXA are induced by HIF early after hypoxia and before any evidence of cell death (Aminova et al., 2005, 2008). Indeed, recent studies indicate that BNIP3 is induced early in hypoxia not to induce death of neurons, but

rather to convert the cell to glycolytic metabolism (away from mitochondrial oxidative phosphorylation) (Zhang et al., 2008). This is accomplished by inducing selective autophagy of mitochondria. Autophagy, or “self-eating,” is a catabolic process involving the lysosomal machinery that allows the degradation of cellular components. It has been shown to be a mechanism to delete aggregated proteins that are not easily digested by the proteasome or to delete damaged or unwanted organelles. In this context, mitochondrial autophagy would facilitate the transition of ischemic neurons to anaerobic metabolism (Semenza, 2008a, 2008b). In the context of SCI, trauma has been shown to induce HIF. HIF is thus poised to transcriptionally upregulate a number of pro-death genes, including BNIP3. BNIP3 can either induce mitochondrial autophagy by a selective signal or via damage to mitochondria (Hamacher-Brady, 2006a, 2006b).

Evidence that autophagy may be a beneficial response after trauma comes from studies that show that small-molecule inducers of autophagy such as rapamycin are protective after injury (Ruan et al., 2008). However, these specific manipulations have yet to be tested in SCI. Whether or not ischemia induces HIF to activate mitochondrial autophagy to convert the spinal cord to anaerobic metabolism, the pro-death effects of HIF-1 can be attributable to its ability to bind to hypoxia response elements in promoters and induce the expression of BH3-only family members BNIP3 (Bcl-2/adenovirus E1B 19kDa interacting protein3 short form), PUMA (p53-upregulated modulator of apoptosis), NIX (BNIP3 Long form), and NOXA (another p53-inducible BH3-only family member; *Noxa* stands for damage) (Aminova et al., 2005, 2008). BNIP3 and PUMA appear to be necessary for ischemia-induced death in neurons; interestingly, PUMA is a more general mediator of oxidative death (Steckley et al., 2007). PUMA appears to be transcriptionally upregulated by a host of oxidants, and its deletion results in neurons that maintain their plasma membrane integrity and their electrophysiologic properties (Steckley et al., 2007). Seminal, pioneering studies by Hsu, Choi, and colleagues showed that global protein synthesis inhibitors such as cycloheximide can inhibit markers of cell death and improve functional recovery (Liu et al., 1997). Although these studies have yet to be replicated, the precise genes that must be upregulated to combat spinal cord injury remain unclear. p53- and/or HIF-dependent upregulation of PUMA or BNIP3 may be important (Kieran et al., 2007; Uo et al., 2007). Alternatively, ischemia leading to c-Jun N-terminal kinase (JNK) activation, Jun phosphorylation and consequent genetic upregulation of Dp5 (a.k.a. Harakiri) may also be important (Yin et al. 2005).

5. EXECUTIONER CASPASES IN THE ACUTE PHASE OF SPINAL CORD INJURY

A discussion about the acute phase of cell death after spinal cord injury would not be complete without some discussion of caspases. Like calpains, caspases are cysteine proteases that can initiate cell death in the cord downstream of death receptors such as Fas or cytokines; alternatively, they can be activated to execute cell death. Evaluation of caspase activity after SCI shows that both “initiator” and “executioner” of these types of caspases are activated. Interestingly, caspase-1 (which converts interleukin-1 beta to its active form) and caspase-3, a marker of the effector phase of classical apoptosis, are observed to be increased in neurons and oligodendrocytes as early as 4 hours after injury (Citron et al., 2008). Other studies have looked at caspase-1, -2, -3, -8, and -9 (see other chapters in this book) and found that only caspase-3, -8, and -9 are activated after traumatic SCI (Beattie et al., 2002b; Colak et al., 2005; Keane et al., 2001; Knoblach et al., 2005; Takagi et al., 2003; Yakovlev & Faden 2001). Although several studies have supported these findings by demonstrating that caspase inhibitors reduce injury and enhance function, there is still great debate over whether these effects are durable and result in functional recovery. Some of the inconsistency in defining caspase activation after SCI may relate to their ability to be nitrosylated and thereby inactivated by peroxynitrite at their active site. Inhibition of caspases by peroxynitrite favors caspase-independent death, described in the preceding section (Lau et al., 2006). Indeed, studies that have measured caspase activation in the acute phase of SCI have used lysates containing buffers with significant concentrations of reducing agents. These conditions might reflect activation of caspases that does not occur in situ. Consistent with this possibility, the findings with molecular or pharmacological deletion of caspases in the acute phase of SCI contrast with studies on molecular deletion of proteins that are involved in mitochondrial release of apoptogenic factors that lead to the effector phase of death. For example, deletion of BH3-only, Bcl-2 family members such as Bax or Puma can lead to neural protection that leads to electrophysiologic or behavioral improvement (Steckley et al., 2007). Other observations also indicate that executioner caspases may not be viable targets for therapy of SCI, as follows.

- (1) Executioner caspases are likely activated after irreversible changes to the mitochondria and other organelles have occurred. Cells preserved by inhibiting downstream caspases are likely dysfunctional in energy homeostasis and synaptic activity; such

dysfunction provides strong pressure to find alternate ways for them to die (Troy & Salvesen, 2002).

(2) Caspases may have other important functions in the CNS, including remodeling and plasticity, and suppressing these functions may be deleterious after SCI (Gilman & Mattson 2002).

(3) Caspases may be important in deleting cancerous and autoimmune cells. Thus prolonged caspase inhibition (>1 month) may present a risk of cancer or autoimmunity (Soengas & Lowe, 2003).

Together, these observations suggest that caspase inhibitors may not be optimal therapeutics. Targeting pathways upstream of caspase activation would be a viable alternative.

6. MITOCHONDRIA AS A TARGET OF SPINAL CORD PROTECTION

A large, compelling body of data indicates that mitochondria are a major checkpoint on several pathways leading to dysfunction and premature death of cells in the spinal cord after injury. Mitochondria appear to link inducers and effectors of cell death pathways by releasing factors that can activate cell death pathways that may be caspase-dependent (cytochrome c, SMAC/DIABLO) and caspase-independent (AIF, endoG). The precise mechanisms by which pro-death factors exit the mitochondria, particularly mitochondria from the spinal cord, remains controversial, but available data suggest that calcium loading of mitochondria and free radical production can cooperate to alter the biology of the mitochondria via the mitochondrial permeability transition (PT) (Maciel et al., 2001). PT is believed to involve the formation of proteinaceous, regulated pores, probably by apposition of inner and outer mitochondrial proteins, which cooperate to form a mitochondrial megachannel. There are a number of metabolic consequences of PT, including the collapse of the mitochondrial membrane potential, release of soluble proteins (cytochrome c, AIF), and overproduction of superoxide ions. Indeed, SOD (superoxide dismutase) overexpression prevents AIF release in motor neurons after SCI (Yu et al., 2006). More recent studies using a non-calcineurin inhibitory cyclosporin A analog reduced mitochondrial dysfunction and tissue damage after traumatic CNS injury (Ravikumar et al., 2007). Novel FDA-approved PT inhibitors have been developed, but the results of some of these agents on contusion injury-induced disability in rodents have been disappointing (Kristal et al., personal communication, March 31, 2011).

As mentioned above, it is possible that once the cell has made the decision to induce PT, it has gone beyond the point of no return. Another approach to the prevention of cell death is to influence those events that precede PT induction, such as calcium overload and free radical generation. Several converging lines of inquiry support the notion that free radicals trigger PT induction and mitochondrial protein release in neurons and possibly oligodendrocytes after SCI (Azbill et al., 1997; Blight & Zimber, 2001; Haghighi et al., 1993). This pathway is also known as the intrinsic pathway to cell death. Over-expression of SOD1 reduced superoxide production and cytochrome c release and delayed motor neuron death after SCI (Sugawara et al., 2002). Superoxide production was observed early after SCI (6 hours) and preceded cytochrome c release and delayed apoptosis (24 hours); superoxide production thus appears to trigger downstream mitochondrial events, possibly through PT induction.

Pharmacological augmentation of glutathione, an important and versatile antioxidant, also reduces makers of oxidative stress, reduces cell damage, and improves functional recovery after SCI (Lucas et al., 2002). These collective findings are particularly intriguing in light of the ability of methylprednisolone to act as an antioxidant (Hall, 1993a, 1993b). However, the effects of methylprednisolone in SCI are modest, have a limited therapeutic window, and have tangible side effects (Blight & Zimber, 2001). Thus novel agents that target antioxidant pathways must be developed for preclinical testing.

In summary, the acute phase of SCI induces cell loss that proceeds downstream of hypoxia-ischemia. Hypoxia-ischemia leads to excitotoxicity, and depending on the amplitude and duration of glutamate receptor activation, necrosis and apoptosis ensue. Apoptosis is mediated by calcium dyshomeostasis and peroxynitrite accumulation, leading to calpain and PARP activation. Increases in both of these enzyme activities can lead to the release of mitochondrial apoptogenic factors that culminate in caspase-dependent or -independent death in neurons or oligodendrocytes (Figure 15-1). Executioner caspases appear to be inhibited by nitrosylation in their active site, favoring non-caspase-dependent pathways. In addition to glutamate receptor stimulation, ischemia can trigger cell death via local increases in peroxynitrite and activation of plasma membrane cation channels (TRPM channels). Alternatively, hypoxia and/or ischemia can directly modulate enzymes such as the prolyl 4 hydroxylases that modulate the stability of transcription factors such as HIF or via stress kinase signaling, c-Jun. These transcription factors appear to act as promoters of pro-death genes such as BNIP3, PUMA,

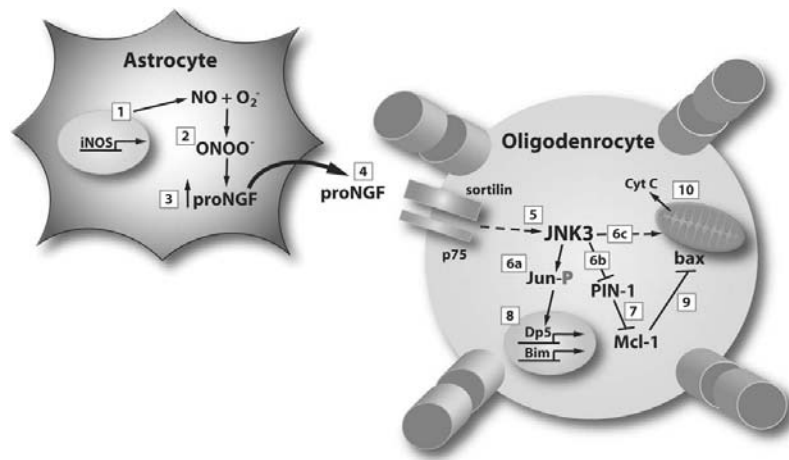


Figure 15-2. Proneurotrophins are produced in a non-cell-autonomous manner, bind to cell surface receptors on neurons (not shown) and oligodendrocytes, and trigger apoptotic death in the subacute phase of SCI. Astrocytes treated with peroxynitrite lead to release of proNGF and binding of this factor to the heterodimeric death receptor p75 and sortilin (Domeniconi et al., 2007). Signaling downstream of this receptor can culminate in mitochondrial cytochrome c release and activation of the apoptosome.

and Harakiri to further stimulate release of apoptogenic factors from the mitochondria.

7. SUBACUTE PHASE: EXTRINSIC PATHWAYS TO DEATH IN NEURONS AND OLIGODENDROCYTES

In the acute phase (minutes to hours), energy failure, inflammation, and excitotoxicity trigger intrinsic pathways to cell death. In the subacute phase (hours to days), extrinsic pathways involving enhanced binding of death ligands to death receptors also appear to be activated after SCI (Casha et al., 2001; Matsushita et al., 2000) (Beattie et al., 2002b; Casha et al., 2001; Matsushita et al., 2000). Death receptors offer another target for neuroprotective therapy. The two death receptors that have received the most attention from the SCI community are the Fas and the p75 neurotrophin receptors and their respective ligands, Fas ligand and pro-nerve growth factor (NGF).

Traumatic SCI leads to the upregulation of the glycoprotein death receptor Fas in apoptotic cells in the gray matter initially after injury and then in oligodendrocytes up to 1 month after injury. Several observations suggest that Fas may initiate apoptosis in gray and white matter after SCI (Casha et al., 2001; Li et al., 2000; Zurita et al., 2001). However, Fas can also be found on microglia and other immune cells after injury. These findings raise the possibility that suppression of Fas-mediated responses could prevent neuronal and/or oligodendroglial death but may potentiate inflammation after SCI (Siegel et al., 2003). Future studies will clarify the net effect of inhibiting Fas pathways in SCI.

An alternative, CNS-specific death receptor and its ligand have also been identified. This death receptor may provide theoretical advantages over Fas ligand as a drug target. Hempstead, working with a number of laboratories, including that of Beattie and Bresnahan, identified a novel function for the unprocessed neurotrophin, proNGF, as a potent mediator of apoptosis for cells that express its receptor, p75, in SCI (Beattie et al., 2002a; Lee et al., 2001). Although the expression of p75 and proNGF is very low in the uninjured adult nervous system, proNGF and p75 are markedly upregulated after SCI and other nervous system injuries, with levels of expression peaking at 3 to 5 days after injury (Beattie et al., 2002a; Harrington et al., 2002; Harrington et al.,

2004). In addition, proNGF is secreted by cells and can be isolated from the cerebral spinal fluid of rodents after injury. Local proNGF secretion at the site of injury acts as a potent apoptotic ligand to promote death of neurons and glia that upregulate expression of p75 within several days of injury (Figure 15-2).

To define potential mechanisms by which proNGF actions can be modulated in vivo, Hempstead and colleagues have sought to identify the receptor complex present on oligodendrocytes and neurons to which proNGF specifically binds to initiate apoptosis (Figure 15-3). Using biochemical cross-linking, they have determined that proNGF recognizes a multimeric complex consisting of the p75 receptor. It also recognizes another transmembrane glycoprotein, sortilin, a VPS (vacuolar protein sorting)-domain containing protein that is expressed both on the cell surface and in sorting vesicles (Hempstead, 2006; Jansen et al., 2007; Massa et al., 2006). Current data suggest that the pro-domain of NGF interacts specifically with sortilin, whereas the mature domain of NGF interacts with p75; importantly, both receptors must be expressed on the cell surface for binding to occur at physiologic (subnanomolar) concentrations. These results suggest that proNGF may initiate signaling by promoting the multimerization of sortilin:p75 receptor complexes. Indeed, agents that interfere with binding of proNGF with this receptor complex impair apoptosis. Addition of antibodies specific for the pro-domain of NGF, or the mature domain of NGF, can reduce apoptosis of neurons and glia expressing both p75 and sortilin by greater than 86% of the level observed when nonimmune immunoglobulin

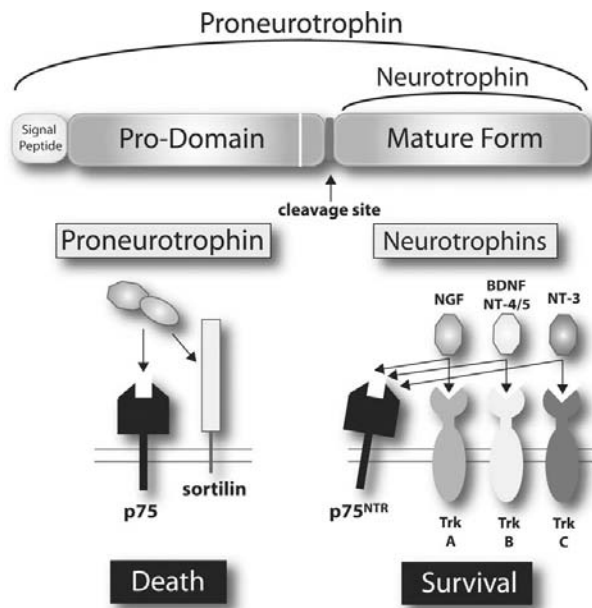


Figure 15-3. Prosurvival and pro-death signaling by neurotrophins. Domain structure of proneurotrophins and their enzymatically processed mature forms.

G was added *in vitro*. *In vitro*, these effects have been shown using cultured neurons or oligodendrocytes. *In vivo*, infusion of the pro-domain or mature domain-specific antisera to mice subjected to CNS injury can block the binding of endogenous, secreted proNGF to p75-containing receptor complexes and can rescue corticospinal neuron death. These results, together with published studies from a host of groups using oligodendroglia, suggest that proNGF is a naturally occurring, pathophysiologic ligand that can initiate apoptosis of neurons and glia in response to injury. Furthermore, agents that impair binding of proNGF to its receptors can result in enhanced survival *in vivo*.

Where is proNGF synthesized in the context of spinal cord injury? Current evidence suggests that proNGF is released from astrocytes or microglia that are “activated” via the intracellular production of peroxynitrite (Hempstead, 2006; Yune et al., 2007; Domeniconi et al., 2007). Activation of astrocytes may result from cytokine or growth factor stimulation (Figure 15-2). Scavengers of peroxynitrite or mitochondrially targeted antioxidants can prevent the release of pro-death factors such as proneurotrophins. Indeed, recent studies suggest that the neuroprotective antibiotic minocycline can inhibit the production of proNGF in microglia to protect oligodendrocytes after spinal cord injury. Of note, minocycline is currently in clinical trials for SCI, but the preclinical results have not been uniformly reproduced between laboratories (Pinzon et al., 2008; Stirling et al., 2004). The results raise the interesting possibility

that the inflammasome in neurons, possibly acting via caspase-1, caspase-11, and other proteins, acts to enhance release of cytokines such as interleukin (IL)-1 β and IL-18. These cytokines can then feedback on microglia or astrocytes to stimulate release of extrinsic apoptogenic factors such as proNGF, Fas, and tumor necrosis factor alpha (TNF- α).

How does binding of proNGF, Fas, or TNF- α to oligodendrocytes induce death? proNGF, Fas, and TNF- α all activate the JNK pathway as a downstream event after binding to their cognate receptors (Figure 15-2). JNK proteins can mediate apoptotic events via two arms. The first arm is a transcriptional one involving the phosphorylation of a number of transcription factors, including possibly c-jun. It appears that more than one JNK isoform (there are three) must be inhibited to prevent death, and as expected, each of the JNKs may have multiple transcription targets. Alternatively, JNKs can regulate the mitochondrial apoptotic pathway directly by facilitating cytochrome c release *in culture*. In oligodendrocytes after SCI, the primary JNK isoform that is activated is JNK3. JNK3 phosphorylates and thereby destabilizes myeloid cell leukemia sequence-1 (Mcl-1) (Li et al., 2007) (Figure 15-2). JNK3 facilitates degradation of Mcl-1 by disrupting its interaction with Pin-1 (phosphorylation specific propyl isomerase never in a mitosis gene a [NIMA] 1). Pin-1 regulates the stability of a number of important mitogen-activated protein kinase substrates, including p53, β -catenin, and the nuclear factor kappa β subunit, p65. In the nervous system, Pin-1 functions primarily as an anti-death protein, as Pin-1-deficient mice develop a progressive neuropathy and tau hyperphosphorylation. Consistent with these findings, Pin-1-deficient oligodendrocytes are more sensitive to apoptosis after spinal cord injury. Mcl-1 represses apoptosis by inhibiting bax function at the mitochondria, downstream of its activation and translocation to the organelle (Germain et al., 2008). Indeed, bax-deficient mice demonstrate durable, enhanced oligodendrocyte survival after spinal cord injury (Dong et al., 2003). Together these studies suggest that extrinsic factors have an important role in triggering delayed oligodendrocyte death.

7.1. Activation of p21 waf1/cip1: Targeting extrinsic and intrinsic pathways to death

Other studies have provided novel insight into the gene expression changes associated with neuronal and oligodendrocyte death in the subacute phase after injury. Twenty-four hours after injury, increased message and protein levels of a cassette of genes that are

associated with cell cycle progression (e.g., E25, c-myc) are founding apoptotic neurons (Di Giovanni et al., 2003). Accordingly, a number of cell cycle inhibitors have been shown to protect neurons from cell death. One protein of particular interest is the cell cycle inhibitor, p21 waf1/cip1. Nuclear p21waf1/cip1 is classically known as a cyclin-dependent kinase inhibitor, but in its cytoplasmic form, it can negatively regulate proapoptotic proteins such as caspases and the upstream kinase, apoptosis signaling kinase-1 (ASK-1) (Coqueret, 2003). Another kinase that is inhibited by p21 that is of particular relevance to SCI and recovery is Rho kinase (Tanaka et al., 2002). The small GTPase RhoA, which has been shown to be upregulated 10-fold after SCI, activates this kinase. Indeed, RhoA inhibitors can reduce the number of TUNEL-labeled cells by 50% after SCI (Dubreuil et al., 2003). This protection is associated with decreased expression of the proneurotrophin receptor, p75. These results predict that agents that can upregulate nuclear p21 waf1/cip1 in the nucleus or cytoplasm should inhibit cell death after SCI through RhoA inhibition, cell cycle inhibition, and caspase inhibition. Agents are currently under development that enhance p21 activity. These agents could inhibit both intrinsic and extrinsic pathways to cell death.

8. CONCLUSION

Traumatic SCI triggers a host of secondary events, including ischemia, excitotoxicity, and inflammatory responses. Each of these processes can induce acute or delayed neuronal or oligodendroglial death. Cell death after SCI comes in many flavors, including classical apoptosis, classical necrosis, parthanatosis, and necroptosis. Autophagy may also contribute or mitigate death. The complexity of death signaling after SCI in neurons and oligodendrocyte underscores the complexity of interventions to reduce cell loss. Single agents that target both extrinsic and intrinsic pathways to death are attracting attention, as well as combinations that act on distinct pathways in distinct cell types. Ultimately, protection of neurons and oligodendrocytes that leads to recovery of function will be the metric by which single or combinatorial interventions can be determined to be successful.

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16

Apoptosis and Homeostasis in the Eye

Jerry Y. Niederkorn

Although the human eye is only a few centimeters in diameter, it contains an extraordinary array of cells and tissues, some of which are found in no other organ (Figure 16-1). The eye is an anatomical extension of the brain and processes an enormously complex array of information that provides us with our most precious sense – our vision. The retinal ganglion cells process more than 500 electrical signals per second, which is roughly equivalent to 10^9 bits of computer information. The conversion of photons of light that enter the eye to crisp visual images in the brain is orchestrated by a diverse array of cells and tissues with vastly different properties and functions. Apoptosis and apoptosis-like processes contribute to the embryonic development of the mammalian eye in the womb and the long-term function of the visual axis from the time of birth to death. The eye, like other components of the central nervous system, is composed of cells that have limited and sometimes no capacity for regeneration. As a result, immune-mediated inflammation can lead to blindness. However, the fluids that fill the eye contain an extraordinary variety of immunosuppressive and anti-inflammatory molecules that control inflammation produced by elements of the innate and adaptive immune systems. Among these eye-derived factors are molecules that induce apoptosis of inflammatory cells. In addition, antigens that enter the eye elicit a unique deviation of the immune response that actively suppresses antigen-specific immune responses such as delayed-type hypersensitivity (DTH), which nonspecifically damages innocent bystander cells within the eye. Interestingly, apoptosis is intimately involved in the induction of this ocular immune deviation.

1. APOPTOSIS AND APOPTOSIS-LIKE PROCESSES THAT SHAPE THE DEVELOPMENT OF THE MAMMALIAN EYE

1.1. Lens

The lens of the mammalian eye grows throughout life, although the growth slows as we age. The lens is composed of two cell types that are derived from ectoderm: the lens epithelial cells and the lens fiber cells. The lens epithelial cells form the outermost layers of the lens and differentiate into lens fiber cells by an apoptosis-like process. Throughout life, the lens epithelial cells differentiate into lens fiber cells, which form the core of the lens. Thus the structure of the lens is somewhat like the layers of an onion, in which the outer layers are composed of the younger lens epithelial cells, and the inner layers are made up of the lens fiber cells that have undergone differentiation, which involves an apoptosis-like process. The differentiation of the lens begins with the elongation of the lens epithelial cells, which subsequently lose their organelles, including the nucleus, mitochondria, Golgi bodies, and endoplasmic reticulum. The nucleus of the lens epithelial cells becomes elongated, and eventually the nuclear membrane disappears and the nucleus itself is no longer distinguishable from the cytoplasmic contents. The denucleation process has many properties reminiscent of apoptosis and involves various regulators that are also involved in classical apoptosis, including members of the caspase family. Studies in the rat have shown that inhibitors of caspase-3-like enzymes block lens differentiation in vitro. Moreover, caspase-3 transcripts are expressed in high amounts in developing lenses in rat eyes. Although various regulators of

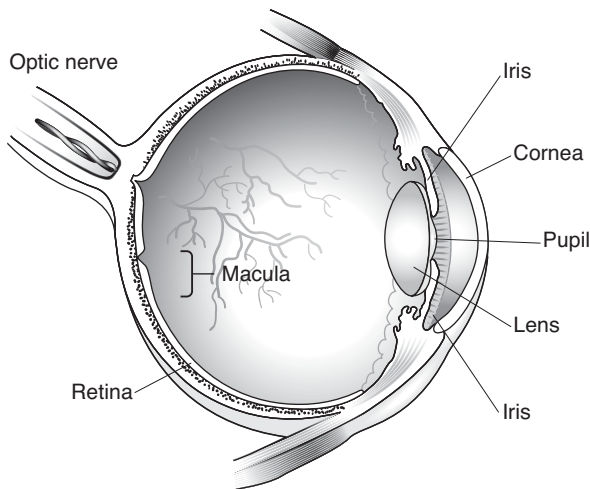


Figure 16-1. Anatomy of human eye. Reprinted courtesy of the National Eye Institute.

apoptosis are expressed in situ in the developing lens, classical apoptosis is not directly involved in denucleation of the lens epithelial cells or in lens development. Normally, apoptotic cells die and are phagocytosed by neighboring cells. By contrast, denucleation culminates in the differentiation of lens epithelial cells into long-lived lens fiber cells that remain throughout the individual's life. During the conversion of the lens epithelial cells to lens fiber cells, the cytoplasm becomes homogenous and accumulates high concentrations of lens-specific proteins, called crystallins, which provide the lens fiber cells and the lens itself with a crystal-clear transparency that is vital for normal vision. The crystallins constitute approximately 90% of the water-soluble proteins of the lens and provide it with its refractile properties.

The lens grows throughout life with the continuous programmed removal of nuclei and other organelles from lens fiber cells. It is noteworthy that in spite of this continuous cell growth and differentiation, spontaneous tumors of the lens (other than experimentally induced neoplasms) have not been described in any species except the cat.

1.2. Retina

Apoptosis plays a pivotal role in the development of the central nervous system (CNS) and the retina. Retinal ganglion cells (RGCs) project from the eye via the optic nerve to the visual centers in the brain. During retinal development, an overproduction of RGCs occurs, with approximately half of the RGCs dying after reaching the visual centers in the brain. One theory holds

that the peripheral neurons compete for a limited supply of neurotrophic factors and that apoptosis is employed to maintain a balance between growth factor supply and neuron demand. Evidence supporting this neurotrophin hypothesis stems from observations showing that removal of one eye promotes the survival of neurons projecting from the other eye to the visual centers in the brain. In rats, up to 90% of the RGCs die during the first postnatal week. In the chick eye, two distinct periods of apoptosis are invoked to shape the development of the RGC population. Apoptosis at an early stage of retinal development is believed to create space for incoming axons of RGC to form the optic nerve. At a later stage of retinal development, apoptosis of RGCs follows innervation and synapse formation with the visual centers in the brain. Emerging evidence suggests that transforming growth factor- β 2 (TGF- β 2) plays a central role in apoptosis of RGCs during retinal development.

2. ROLE OF APOPTOSIS IN DISEASES OF THE EYE

2.1. Glaucoma

It has been estimated that by the year 2020, almost 80 million people will have glaucoma, which is one of the leading causes of irreversible blindness. Glaucoma is a disease of the optic nerve. A common misconception is that glaucoma is produced by chronic elevation in intraocular pressure. Although glaucoma is often associated with increased intraocular pressure, a significant number of individuals experience glaucoma even though their intraocular pressures are within the normal ranges (i.e., normal pressure glaucoma). A significant body of evidence indicates that ocular hypertension alone is neither necessary nor sufficient for the production of optic neuropathy. It has been proposed that in some cases, glaucoma is an immune-mediated disease. Some glaucoma patients produce antibodies directed at heat shock proteins (HSPs), such as HSP60 and HSP27, which are upregulated in glaucomatous optic nerve heads. It has been proposed that HSPs have important neuroprotective properties, but under pathological conditions they can serve as immunogens that provoke adaptive immune responses that culminate in the generation of autoantibodies against retinal antigens. Antibodies directed against HSP27 have been detected in the sera of glaucoma patients, and when tested in vitro, these sera induce RGC death via an apoptotic mechanism. Animal studies have recently demonstrated that immunization with HSP27 and HSP60 results in optic neuropathy that is characterized by a T-cell infiltrate and apoptosis of

RGC. T cells isolated from HSP60 and HSP27 immunized rats induced apoptosis of RGC cells in vitro by a process characteristic of apoptosis. Moreover, HSP-immune T cells elaborated a soluble factor that induced apoptosis of the Fas⁺ RGC cells in a cell contact-independent manner, which could be blocked with antibody specific for FasL. Additional studies demonstrated that recombinant human FasL alone induced apoptosis of rat RGC cells in vitro, suggesting that the generation of anti-RGC T cells was antigen-specific, but the effector mechanism that culminated in apoptosis of RGC cells was antigen non-specific. Although these findings are provocative, they do not account for all cases of glaucoma, and competing hypotheses suggest that other conditions such as neurotrophin deprivation can be key factors in the pathogenesis of glaucoma.

2.2. Age-related macular degeneration

Age-related macular degeneration (AMD) is the leading cause of blindness in the elderly in developed countries and affects approximately 35% of individuals 75 years of age or older. AMD is characterized by progressive degeneration of the photoreceptors and retinal pigment epithelial (RPE) cells in the small central portion of the retina called the macula, which is responsible for high visual acuity (Figure 16-1). The growth of new blood vessels in the choroid layer (i.e., choroidal neovascularization; CNV), which lies beneath the retina, develops in 10% of AMD patients yet accounts for 90% of the blindness in AMD (Figure 16-2). Macrophages have been implicated in the etiology of AMD, with some studies suggesting that macrophages stimulate CNV, whereas other studies indicate that they inhibit CNV. Investigations in a mouse model of laser-induced CNV have provided evidence that CNV is the result of an imbalance in FasL-mediated apoptosis of newly generated choroidal vascular endothelial cells. Evidence suggesting that FasL-induced apoptosis might regulate CNV has arisen from studies on ocular biopsies from AMD patients, which demonstrated that new choroidal blood vessels in AMD patients expressed Fas receptor. Investigations in a mouse model of laser-induced CNV indicated that CNV was significantly increased in mice with defective, nonfunctional FasL (*gld/gld*) and in Fas receptor-deficient mice (*lpr/lpr*). Studies from interleukin (IL)-10 knockout (KO) mice revealed that the absence of IL-10 resulted in polarization of intraocular macrophages to an antiangiogenic phenotype. As its name implies, AMD is a disease of senescence. Interestingly, the ability of laser-induced injury to stimulate CNV increases as mice age, and the level

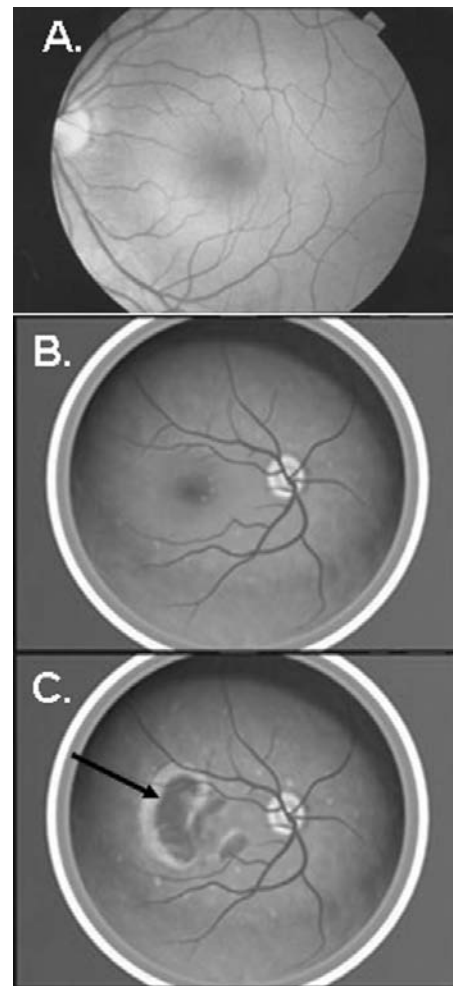


Figure 16-2. Retinal neovascularization in age-related macular degeneration (AMD). A. Normal retina. B. Early AMD with retinal neovascularization. C. AMD with retinal neovascularization and degeneration of macula (arrow). Reprinted courtesy of the National Eye Institute. See Color Plate 16.

of IL-10 in the posterior compartment of the eye is highest in older mice. Moreover, FasL expression on macrophages diminishes with age. Macrophages from aged mice have reduced expression of FasL and demonstrate a diminished capacity to inhibit vascular endothelial cell growth. These findings have led to the hypothesis that resident macrophages in the eye are crucial for maintaining a normal level of choroidal vascularization, and that as we age, the level of FasL diminishes on ocular macrophages and the concentration of immunosuppressive cytokines, such as IL-10, in the intraocular milieu increases. Macrophage function is influenced by the microenvironment, and as such, macrophages can behave either as either proangiogenic or antiangiogenic effectors. However, in the aging eye, the combination of elevated IL-10 and diminished FasL tilt the ocular macrophage population to a proangiogenic phenotype.

3. APOPTOSIS AND SURVEILLANCE OF INTRAOCULAR TUMORS

The immune privilege of the eye has been recognized for more than 140 years and is known to permit the long-term survival of tissue and tumor allografts. However, some murine tumors transplanted into the anterior chamber circumvent immune privilege and undergo immune rejection in the eye. A number of theories have been offered to explain the circumvention of ocular immune privilege. Among these theories is the notion that some tumors succumb to apoptosis induced by cell membrane molecules that are expressed on intraocular cells. The cells lining the interior of the eye are decorated with a variety of molecules that disable immune effector elements and in some cases, can also induce apoptosis of intraocular tumor cells. These include FasL, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), programmed death ligand-1 (PD-L1), and complement regulatory proteins. However, at least one of these molecules (TRAIL) can also purge the eye of tumor cells bearing its apoptosis-inducing receptor (DR5). TRAIL is expressed on a wide variety of ocular cells and is regulated by interferon- γ (IFN- γ), as its expression is virtually absent in the eyes of IFN- γ KO mice. Lee and coworkers reported that P815 tumor cells not expressing TRAIL receptor 2 (DR5) grew progressively in the eyes of allogeneic BALB/c mice. However, the same tumor cells underwent brisk rejection if they were transfected with TRAIL-R2 (DR5) cDNA to induce DR5 expression. Moreover, in vitro studies testing a variety of DR5⁺ tumor cell lines demonstrated that DR5⁺ tumor cells underwent apoptosis when incubated in vitro with ocular cells that constitutively expressed TRAIL. Apoptosis could be blocked with an antagonistic anti-TRAIL antibody, thereby confirming the specificity of the TRAIL-induced tumor cell apoptosis by ocular cells. Although these results involved TRAIL-induced apoptosis of allogeneic and xenogeneic tumors, it is noteworthy that in a follow-up study, more than half of the human ocular melanoma cell lines tested expressed TRAIL-R2 and were susceptible to TRAIL-induced apoptosis. Thus human ocular tumors express TRAIL-R2 and are susceptible to TRAIL-induced apoptosis. It remains to be confirmed whether this constitutes an effective immune surveillance mechanism for intraocular tumors.

4. APOPTOSIS AND OCULAR IMMUNE PRIVILEGE

The immune privilege of the eye is the sum total of unique anatomical, physiologic, and immunoregulatory processes that block the induction and expression of

both innate and adaptive immune effector mechanisms. The blood vessels of the anterior segment of the eye are non-fenestrated and create a blood:ocular barrier that limits the extravasation of circulating leukocytes into the eye. Leukocytes that succeed in entering the anterior chamber encounter aqueous humor, which contains a potpourri of anti-inflammatory and immunosuppressive molecules. Among these is a 10-kDa peptide that induces apoptosis of natural killer cells, T cells, neutrophils, and macrophages. Leukocytes that escape apoptosis mediated by aqueous humor-borne factors are greeted by at least three different cell membrane-bound molecules – FasL, TRAIL, and PD-L1 – that are expressed on cells lining the interior of the eye and have been shown to induce apoptosis of activated T lymphocytes.

One of the remarkable manifestations of ocular immune privilege is the exceptionally high survival rate for corneal allografts. In uncomplicated first-time cases, corneal allografts experience a 90% acceptance rate, even though HLA-matching is not employed and systemic immunosuppressive drugs are not used. Studies in rodents have demonstrated that corneal cells express both FasL and PD-L1, which induce apoptosis of activated T lymphocytes and whose expression is crucial for corneal allograft survival.

The immune privilege of the anterior chamber (AC) of the eye is also maintained by a dynamic immunoregulatory process that downregulates T-cell-based inflammation in an antigen-specific manner. That is, antigens introduced into the AC of the eye induce an immune deviation that suppresses T-cell-mediated immunity such as DTH and cytotoxic T-lymphocyte (CTL) activity while preserving antibody responses. This anterior chamber-associated immune deviation (ACAID) is associated with the immune privilege of tumor allografts transplanted into the AC of the eye and corneal allografts transplanted over the AC. ACAID is a complex immunoregulatory phenomenon that is initiated when antigens are introduced into the AC and involves the eye, thymus, spleen, and sympathetic nervous system. FasL-induced apoptosis appears to be intimately involved in the induction of ACAID. Early evidence that FasL-induced apoptosis was involved in ocular immune privilege in general and ACAID in specific arose from experiments in which herpes simplex virus-1 (HSV-1) was injected into the AC of mice. Injection of HSV-1 into subcutaneous sites elicits robust HSV-specific DTH. However, HSV-1 injected into the AC induces ACAID, as demonstrated by the downregulation of HSV-1-specific DTH. However, AC injection of HSV-1 into mice with defective Fas receptor (*lpr*) or FasL (*gld*) not only fails to induce ACAID, but in fact, stimulates positive

HSV-1-specific DTH and provokes intense ocular inflammation. These results suggest that FasL-induced apoptosis is necessary for the induction of immune deviation (i.e., ACAID) and that the absence of FasL/FasR interactions robs the anterior chamber of its immune privilege. This was confirmed in additional experiments involving hapten-derivatized spleen cells. Syngeneic spleen cells treated with the hapten trinitrophenol (TNP-spl) will induce TNP-specific DTH responses when injected subcutaneously, whereas the same TNP-spl injected into the AC induce inhibition of TNP-specific DTH. However, if spleen cells from Fas receptor-deficient mice are derivatized with TNP and injected into the AC, they fail to induce ACAID and instead elicit TNP-specific DTH. Likewise, when TNP-derivatized spleen cells from normal mice are injected into the eyes of FasL-deficient mice, ACAID is not induced, thereby confirming that FasL-induced apoptosis of TNP-derivatized cells is crucial for the induction of ACAID. Although these findings indicate a clear role for FasL-induced apoptosis in the induction of ACAID, apoptosis induced by other means can also promote the induction of ACAID. That is, TNP-derivatized spleen cells from Fas receptor-deficient mice treated with x-irradiation undergo apoptosis and when injected into the AC, will induce ACAID as effectively as TNP-derivatized spleen cells from wild-type mice. Follow-up studies demonstrated that apoptosis, elicited by either FasL or x-irradiation, stimulated the rapid production of IL-10, which altered the behavior of antigen-presenting cells and rendered them tolerogenic.

5. CONCLUSIONS

Apoptosis contributes to the development of the mammalian eye in utero and is a crucial element in the homeostasis of the visual axis from birth to death (Figure 16-3). Although apoptosis is necessary for the maintenance of vision, it also has a dark side and is a key element in the pathogenesis of the two leading causes of blindness, glaucoma and AMD. It is widely believed that regulation of ocular inflammation is critical for preserving vision, as many ocular cells cannot regenerate, and immune-mediated injury to these cells would culminate in blindness. Apoptosis is a central process in the

Retinal macrophages express FasL and regulate retinal blood vessel development by inducing apoptosis of Fas⁺ vascular endothelial cells.

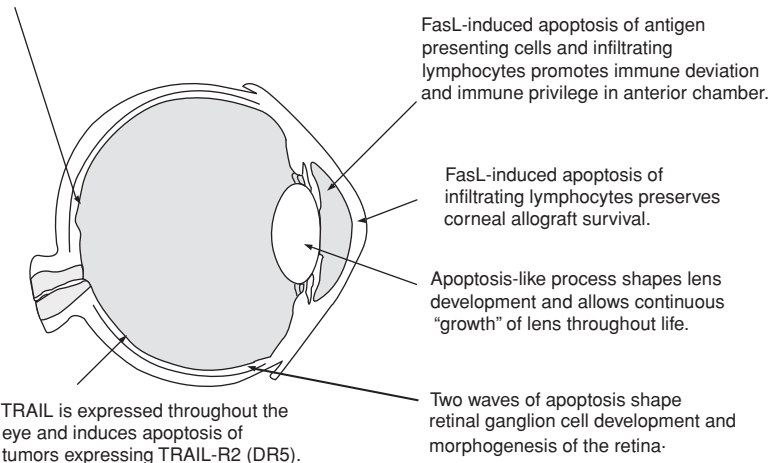


Figure 16-3. Apoptosis shapes morphogenesis of the eye and sustains immune privilege by multiple mechanisms. Reproduced with permission from Nature Publishing Group; *Journal of Investigative Dermatology Symposium Proceedings* 8:168, 2003.

maintenance of ocular immune privilege and in restraining ocular inflammation. However, failure of ocular immune privilege can have devastating consequences. Indeed, the three leading causes of infectious blindness – trachoma, river blindness, and HSV keratitis – are immune-mediated diseases in which the unrestrained, chronic immune response to ocular pathogens, rather than the direct cytopathic effects of the pathogens, is the primary cause of blindness.

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17

Cell Death in the Inner Ear

Lisa L. Cunningham and Justin Tan

The inner ear transduces sound energy and head motion into neural impulses. These signals are detected by six sensory patches in the fluid-filled spaces of the inner ear. The snail-shaped cochlea detects sound, whereas the vestibular system serves balance and gravity-detection functions. All six sensory patches in the inner ear use mechanosensory hair cells to transduce fluid motion signals into neurotransmitter release. These sensory cells are sensitive to death from noise trauma, aging, and certain therapeutic drugs. Hair cells in nonmammalian vertebrates are regenerated after they die, resulting in functional recovery of hearing and balance. In contrast, mammalian sensory hair cells are not regenerated, and their loss results in permanent hearing and/or balance disorders. Cochlear hair cells make synaptic connections with spiral ganglion neurons. Spiral ganglion neurons are bipolar cells with dendrites that synapse with the basal surfaces of hair cells and axons that comprise the eighth cranial nerve. Hair cells provide trophic support to spiral ganglion neurons. Therefore, death of hair cells is often followed by spiral ganglion neuron degeneration. Hearing loss is the most common sensory impairment in humans and is the sixth most common chronic health problem in the United States. This chapter addresses apoptotic death of sensory hair cells in response to ototoxic drugs and the subsequent death of spiral ganglion neurons (SGNs).

1. HAIR CELLS ARE THE SENSORY RECEPTOR CELLS IN THE HEARING AND BALANCE ORGANS OF THE INNER EAR

The mechanosensory hair cell is a polarized cell characterized by a bundle of rigid stereocilia embedded in the apical surface. These stereocilia project into the fluid of the endolymphatic compartment. Endolymph is a unique extracellular fluid that is high in potassium,

which is secreted into the endolymphatic space by the stria vascularis. When sound waves travel through the ear canal and into the middle ear space, the three bones of the ossicular chain transmit this airborne energy into fluid motion in the inner ear (Figure 17-1). The fluid motion results in deflection of the stereocilia bundles on the apical surfaces of hair cells in the cochlear organ of Corti. This deflection opens mechanically gated ion channels in the stereocilia and allows cations (primarily K^+ and Ca^{2+}) from endolymph to enter the hair cell. The influx of cations results in a receptor potential that causes opening of voltage-gated Ca^{2+} channels, triggering the release of glutamate from the basal surface of the inner hair cell. The neurotransmitter enters the synaptic cleft and activates receptors on afferent projections of spiral ganglion neurons. In the vestibular system, the three semicircular canals are oriented at right angles to one another and serve to detect angular acceleration of the head. Each semicircular canal contains a sensory epithelium called the *crista ampullaris*, which contains hair cells that are activated by the movement of fluid in the semicircular canals as the head moves. This allows for detection of angular head movement in three dimensions. The vestibule of the ear is between the semicircular canals and the cochlea, and it contains the maculae of the utricle and saccule, which are oriented at right angles to one another. The utricle detects linear acceleration, whereas the saccule detects gravity. The utricle and saccule are both otolithic organs, meaning that the stereocilia are coupled to a mass (called otoconia). When the otoconia move in relation to the hair cells, the stereocilia are deflected and the cell releases neurotransmitter. Together, these six organs (the organ of Corti, utricle, saccule, and three semicircular canals) comprise the sensory receptor portions of the inner ear (Figure 17-1).

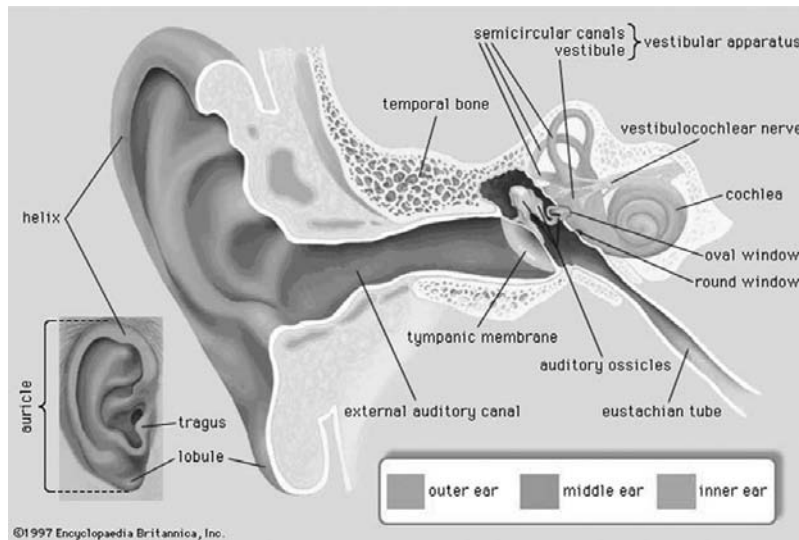


Figure 17-1. The ear. The ear comprises three portions. The outer ear includes the external ear, ear canal, and the tympanic membrane. The middle ear is an air-filled space containing the auditory ossicles (malleus, incus, and stapes). The inner ear is a fluid-filled space that includes both the vestibular organs (three semicircular canals and the utricle and saccule in the vestibule) and the hearing organ (cochlea). The vestibulocochlear nerve is divided into the vestibular portion and the spiral ganglion portion, which innervates the cochlea. Reprinted with permission from *Encyclopaedia Britannica*, © 1997 by Encyclopaedia Britannica, Inc. See Color Plate 17.

2. HAIR CELLS SYNAPSE WITH VESTIBULAR GANGLION NEURONS AND SPIRAL GANGLION NEURONS

Hair cells in the six sensory patches of the inner ear make synaptic connections with bipolar first-order neurons of the eighth cranial nerve. The vestibular hair cells are innervated by the vestibular ganglion portion of the nerve, and the organ of Corti is innervated by the SGNs. SGNs are primary auditory neurons that make synaptic connections with inner and outer hair cells of the organ of Corti and thus serve as afferent neurons from the peripheral organ of Corti to the cochlear nuclei in the central auditory system. In many cases, SGN survival is intimately linked to the pathological status of sensory hair cells, which provide trophic support for SGNs.

3. THE COCHLEA IS THE HEARING ORGAN

In the cochlea, two types of hair cells are contained within the organ of Corti. A single row of inner hair cells and three rows of outer hair cells run the length of the organ of Corti from base to apex (Figure 17-2). Inner hair cells are much more densely innervated than outer hair cells. A single inner hair cell is innervated by ≥ 10 heavily myelinated afferent nerve fibers, most of which are contacted by a small efferent fiber. In contrast, a single unmyelinated afferent fiber will innervate many outer hair cells. Thus inner hair cells are primarily responsible

for sound transduction. Outer hair cell receptor potentials result in length changes of the outer hair cells along the length of their cell bodies. This “electromotive force” serves to mechanically amplify sound energy transmission to the inner hair cells by 50 to 70 decibels. Outer hair cells are present only in mammalian inner ears, and they increase the range of frequencies that can be detected, as well as the frequency selectivity of the mammalian inner ear relative to that of birds or reptiles. In response to damaging stimuli such as noise or ototoxic drug exposure, outer hair cells are much more susceptible to death than inner hair cells.

Because of a mechanical compliance gradient, the organ of Corti is arranged tonotopically such that the base of the cochlea detects high-frequency sounds, and lower frequency sounds are detected more apically (Figure 17-2).

The hair cells arranged along this tonotopic map are each responsive to a fairly narrow range of frequencies, and each hair cell has a characteristic frequency at which it is most sensitive. This frequency selectivity is largely a result of the properties of the basilar membrane underlying the organ of Corti. This membrane vibrates in response to fluid motion in the cochlea. The basilar membrane has gradients of both stiffness and width (narrow and stiff at the base; wide and compliant at the apex) that cause different regions of the membrane to vibrate differentially according to the frequency of the stimulus. The ossicles in the middle ear transmit sound energy into the fluid of the inner ear, driving the motion of the basilar membrane as a traveling wave that moves from base to apex and results in maximum basilar membrane motion at the region responsive to the frequency of the stimulus. Because the traveling wave moves from base to apex, there is some displacement of regions of the basilar membrane that are more basal than the region maximally displaced, so there is also a lesser stimulation of higher-frequency hair cells. Traumatic noise exposure can result in death of hair cells that are most responsive to the frequencies contained in the traumatic stimulus. In addition, hair cells that are located more basal than the region of maximum basilar membrane displacement (i.e., higher-frequency hair cells) can also be damaged or killed.

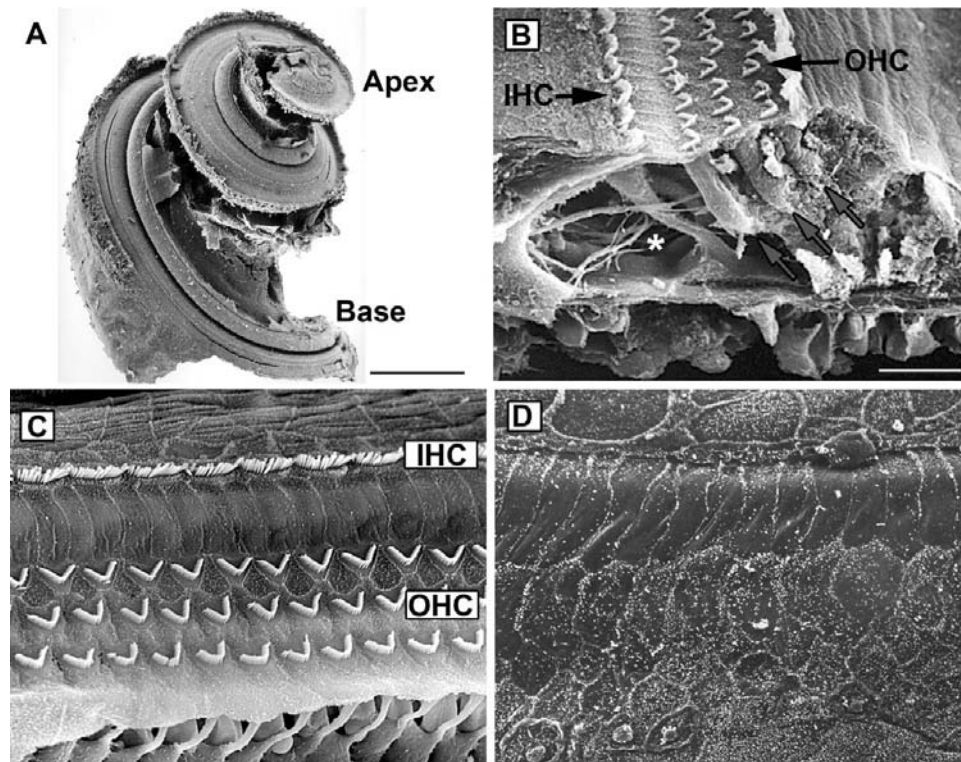


Figure 17-2. The cochlea. **A.** Scanning electron micrograph of an adult rat cochlea with the bony labyrinth dissected away. The cochlea spirals from base to apex, with sensory hair cells along the entire length. The base of the cochlea detects higher-frequency sounds, whereas the apex is more sensitive to low frequencies. **B.** Cross-section of a rat cochlea. Three rows of v-shaped outer hair cell (OHC) stereocilia bundles and a single row of inner hair cell (IHC) stereocilia can be seen at the upper center of the photo. Blue arrows point to outer hair cell bodies. Asterisk indicates the location of the tunnel of Corti, and green arrows point to efferent nerve processes (top arrow) and spiral ganglion fibers (lower arrow). **C.** Surface of the rat organ of Corti showing three rows of outer hair cell (OHC) stereocilia and a single row of inner hair cell (IHC) stereocilia. **D.** Cochlea of a rat treated with the aminoglycoside antibiotic amikacin. Both inner and outer hair cells at the base of the cochlea are missing. Scale bar in A = 2 mm. Scale bar in B = 20 μm . Figures reprinted with permission from Rémy Pujol and Marc Lenoir, INSERM, France. See Color Plate 18.

3.1. Ototoxic hair cell death

Hair cells are sensitive to death from aging, noise trauma, and exposure to certain therapeutic drugs. Although this chapter focuses on mechanisms of hair cell death in response to ototoxic drug exposure, there is significant evidence that ototoxic hair cell death shares common molecular and signal transduction pathways with hair cell death caused by noise exposure and even aging. Two major classes of drugs have been identified that result in death of sensory hair cells in the inner ear. These ototoxic drugs are the aminoglycoside antibiotics and the anti-neoplastic agent cisplatin. Hair cell death in response to aminoglycosides and cisplatin will be discussed in more detail later in this chapter, but some important similarities exist between them in terms of their toxicity. First, both aminoglycosides and cisplatin are also nephrotoxic, and their ototoxic and nephrotoxic side effects are dose-limiting for both drugs. Second, both aminoglycosides and cisplatin cause death of outer hair cells in the base of

the cochlea, resulting in a high-frequency sensory hearing loss. With continued exposure or increased doses, either aminoglycosides or cisplatin will result in progressively more apical (low-frequency) hair cell death. Both classes of drugs result in permanent hearing loss in a significant portion of humans receiving these drugs. Both drugs can also cause death of vestibular hair cells, resulting in permanent balance disorders.

3.2. Aminoglycoside-induced hair cell death

The aminoglycoside antibiotics were first discovered in the 1940s, and they remain among the most commonly used antibiotics worldwide. This class of antibiotics includes gentamicin, neomycin, amikacin, tobramycin, kanamycin, streptomycin, and paromomycin. Aminoglycosides are small (300–600 Dalton) molecules consisting of several saturated 6-carbon rings with attached amino groups. They have broad-spectrum bactericidal properties, especially against Gram-negative bacteria.

Their primary antibacterial action is thought to be binding to the bacterial 16S ribosomal RNA and inhibiting protein translation. Aminoglycosides are very effective against *Mycobacterium tuberculosis* and are useful in the treatment of drug-resistant tuberculosis. They are also commonly used to treat *Pseudomonas* infections, including the recurrent *Pseudomonas* infections commonly seen in cystic fibrosis patients.

Shortly after their discovery, aminoglycosides were reported to have both ototoxic and nephrotoxic side effects. Sensory hair cells are the primary targets of aminoglycoside ototoxicity (Figure 17-2). Aminoglycosides are toxic to sensory hair cells in all species that have been examined, including aquatic vertebrates, reptiles, birds, and mammals. There is evidence that aminoglycosides enter sensory hair cells via the mechanotransduction channel itself. Once inside the hair cell, aminoglycosides accumulate in lysosomes. Transmission electron microscopic studies of hair cells treated with aminoglycosides have revealed early increases in the number of lysosomes, numerous vacuoles in the cytoplasm, and mitochondrial swelling and cristae disorganization. Scanning electron microscopic observations range from disorganization and fusion of stereocilia at low doses of aminoglycosides to complete loss of the hair cell at higher doses.

One proposed mechanism of aminoglycoside-induced hair cell death is the formation of reactive oxygen species (ROS) in the hair cell. Gentamicin has been shown to form a complex with iron that catalyzes the generation of free radicals, including superoxide, which can then lead to formation of the hydroxyl radical via iron-catalyzed Fenton reactions. The role of ROS formation in aminoglycoside-induced hair cell death is supported by evidence that ototoxicity is inhibited by both iron chelators and free radical scavengers, including glutathione.

Aminoglycoside-induced hair cell death has been characterized as apoptotic by both morphologic and molecular studies. Both cisplatin-induced and aminoglycoside-induced hair cell death are significantly inhibited by broad-spectrum inhibition of caspases. Experiments using fluorescent peptide caspase substrates have indicated that caspase-9 is a major mediator of aminoglycoside-induced hair cell death. Caspase-9 activation requires release of mitochondrial cytochrome c and apoptosome formation that results in autoactivation of caspase-9. Once activated, caspase-9 activates caspase-3, a major executioner caspase that carries out the apoptotic program by cleaving proteins essential for cellular survival. Experiments using small-molecule inhibitors of X-linked inhibitor of apoptosis protein

(XIAP) suggest that XIAP may inhibit gentamicin-induced activation of caspase-3 in hair cells. Overexpression of Bcl-2 also inhibits aminoglycoside-induced hair cell death and caspase-9 activation.

Aminoglycoside-induced hair cell death is mediated by activation of c-Jun NH₂-terminal kinases (JNKs). JNKs are mitogen-activated protein kinases (MAPKs) that are activated in response to a variety of stresses, including inflammatory cytokines, osmotic stress, radiation, and excitotoxicity. Aminoglycosides result in phosphorylation of JNKs in hair cells. Inhibition of JNK signaling using a variety of approaches can protect hair cells against aminoglycoside toxicity. Activation of JNK is upstream of caspase-9 activation in hair cells. Interestingly, JNK inhibition does not protect against cisplatin-induced hair cell death, indicating that although aminoglycoside- and cisplatin-induced ototoxicity share common downstream molecular mechanisms, their upstream signaling mechanisms diverge.

3.3. Cisplatin-induced hair cell death

Cisplatin is among the most effective and widely used chemotherapeutic drugs available, and it is used worldwide to treat a broad range of tumors, including testicular, bladder, lung, stomach, and ovarian cancers. Estimates of the incidence of hearing loss in patients receiving cisplatin range from 12% to 90%, depending on the diagnosis, total dose of cisplatin received, and population examined. Cisplatin exposure results in damage or death of several cell types in the inner ear, including auditory and vestibular hair cells, stria vascularis, and spiral ganglion cells.

In cancer cells, cisplatin binds to DNA and results in DNA cross-linking that induces apoptosis via both p53-dependent and p53-independent mechanisms. The tumor suppressor p53 mediates cellular responses to DNA damage via transcriptional upregulation of genes that regulate cell cycle checkpoints as well as apoptosis. In addition, p53 can regulate cell fate via transcription-independent mechanisms. For example, p53 may promote cytochrome c release both indirectly by promoting the translocation of the proapoptotic Bcl-2 family protein Bax to the mitochondria and directly by translocating to mitochondria under apoptotic conditions. Studies in the auditory system have revealed that platinated DNA is present in most cells of the organ of Corti as well as in the stria vascularis in guinea pigs after exposure to cisplatin. One study reported that the p53 inhibitor pifithrin alpha inhibits cisplatin-induced hair cell death and caspase activation.

Like aminoglycosides, cisplatin results in the formation of reactive oxygen species in hair cells, including superoxide. Some thiol antioxidants, including sodium thiosulfate, D-methionine, and lipoic acid, can inhibit cisplatin-induced ototoxicity. However, some of these thiols, including sodium thiosulfate, diminish cisplatin's tumoricidal activity by the formation of inactive platinum–thiol conjugates.

3.4. Therapeutic strategies to prevent hair cell death

Several cotherapies have been shown to inhibit ototoxic hair cell death in animal model systems. As mentioned previously, a variety of antioxidants can inhibit both aminoglycoside- and cisplatin-induced hair cell apoptosis. Similarly, ototoxicity is inhibited by either inhibition of caspase activity or upregulation of antiapoptotic Bcl-2 family members. Interest is emerging in intrinsic protective mechanisms in the inner ear that, if activated, may be able to inhibit hair cell death. One such intrinsic protective mechanism is the activation of heat shock proteins (HSPs). HSP induction is one of the most ubiquitous and highly conserved stress responses in biology. Stress-induced HSP expression promotes cellular survival in a large number of systems, and HSPs can directly inhibit apoptotic signaling. One well-characterized HSP inducer is heat stress, which induces most HSPs and protects cells against a number of stresses. For example, short-term total-body hyperthermia has been shown to protect the retina against light-induced damage and to prevent ischemia-induced death in both cardiomyocytes and hippocampal neurons. Induction of HSPs via either total-body hyperthermia or local hyperthermia inhibits noise-induced hearing loss. Induction of HSPs via heat shock inhibits both cisplatin- and aminoglycoside-induced hair cell death in vitro. Hsp70 is both necessary and sufficient to account for this protective effect of heat shock against aminoglycoside-induced hair cell death. Geranylgeranyl acetone, a chemical HSP inducer, inhibits aminoglycoside-induced hair cell death in vitro. Constitutive over-expression of Hsp70 in transgenic mice inhibits aminoglycoside-induced cochlear hair cell death and hearing loss. It is likely that clinical strategies aimed at inhibiting ototoxic hearing loss will involve both inhibition of apoptotic signaling and upregulation of intrinsic protective mechanisms, possibly including HSP induction.

3.5. Challenges to studies of hair cell death

Both aminoglycosides and cisplatin result in death of sensory hair cells in the inner ear. This death is

inhibited in animal studies by several cotherapies, including antioxidants and caspase inhibition. However, there is currently no commonly used cotherapy for the prevention of either cisplatin- or aminoglycoside-induced hair cell toxicity. Studies of apoptosis in the inner ear are complicated by a lack of suitable model systems in which to examine apoptotic signaling. Although several cell lines have been developed from inner ear tissue, no cell line has yet been identified that develops morphological features of hair cells (i.e., stereocilia) and none that is sensitive to both aminoglycoside- and cisplatin-induced death. Furthermore, adult mammalian cochlear hair cells do not survive in culture for more than a few hours. Therefore, research into sensory hair cell apoptosis is usually carried out in whole organ cultures, either of the organ of Corti from neonatal rodents or in the macular organs (utricle and/or saccule) from mature rodents. Both of these systems have limitations. First, both yield a mixture of cell types that includes hair cells, supporting cells, stromal cells, and neuronal processes. This nonhomogeneity of the culture makes it difficult to be certain that changes observed by quantitative methods such as real-time reverse-transcriptase polymerase chain reaction and Western blotting occur in the hair cells themselves. Second, results obtained in cultures from neonatal animals may not reflect the degenerative changes that occur in mature hair cells, and differences also may exist between cochlear and vestibular hair cells in their responses to stress. Third, both systems yield very small numbers of hair cells: the mouse organ of Corti and the adult mouse utricle each contain only approximately 3,000 hair cells. This is an extremely small amount of tissue, and this limitation restricts the feasibility of many biochemical and molecular biology techniques that require large numbers of cells. Despite these limitations, significant progress has been made in recent years toward understanding the mechanisms underlying sensory hair cell death and survival.

4. SPIRAL GANGLION NEURON DEATH

When hair cells are destroyed by noise trauma or ototoxic drugs such as aminoglycoside antibiotics and cisplatin, the SGNs show signs of apoptosis within days and continue to degenerate over a lengthy period of time (Figure 17–3). In certain cases, genetic mutations that cause atrophy of the organ of Corti also lead to a corresponding loss of SGNs. Emerging evidence supports the concept that cells lying close to inner and outer hair cells complement the function of the sensory hair cells in promoting SGN survival.

4.1. Neurotrophic support from sensory hair cells and supporting cells

Neurotrophins are a major family of molecules that are required for SGN development and survival. The neurotrophins – nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), and neurotrophin 4/5 – constitute a family of secreted molecules that provide target-derived guidance cues for neurons and are essential for neuronal survival and function. During development, inner and outer hair cells as well as supporting cells of the organ of Corti express BDNF, NT3, and another type of neurotrophic factor called glial cell-derived neurotrophic factor (GDNF) to varying degrees and in a spatio-temporal pattern. The importance of BDNF and NT3 in inner ear development and SGN survival is demonstrated by knockout mouse models in which deletion of these neurotrophic genes in mice results in both loss of SGNs and retraction or retardation of their peripheral processes to the organ of Corti. Thus BDNF and NT3 can function as target-derived factors to regulate the survival of SGNs and guide their innervation to hair cells in the organ of Corti during development. Recently, it has been suggested that neurotrophin expression in sensory hair cells and supporting cells is increased by another trophic signaling mechanism involving neuregulins produced by SGNs and their receptors, *erbB2*, present in hair cells. This reciprocal signaling between SGNs and hair cells is thought to increase neurotrophin expression in either supporting cells or hair cells. Consistent with this reasoning, it has been demonstrated that transgenic mice with disrupted *erbB* signaling demonstrate dramatic loss of SGNs and reduced NT3 expression.

Because neurotrophins need to bind to their cognate receptors to mediate survival, it is reasonable to speculate that mice deficient in these receptors would show corresponding SGN loss. Among these receptors, the tropomyosin-related kinase (Trk) receptor tyrosine kinase B binds selectively to BDNF, whereas TrkC preferentially interacts with NT3. These receptors are expressed in SGNs, and mice deficient in TrkB or TrkC show significant SGN loss and innervation defects at the organ of Corti. Furthermore, TrkB and TrkC double knockout mice display an even more severe SGN loss than either of the single knockout mouse models, underscoring the requirement of both neurotrophins for SGN survival. The importance of these neurotrophins in the inner ear does not appear to be restricted to this developmental window, because sensory hair cells and supporting cells of the adult organ of Corti continue to express BDNF and NT3. In addition, TrkB and TrkC expression

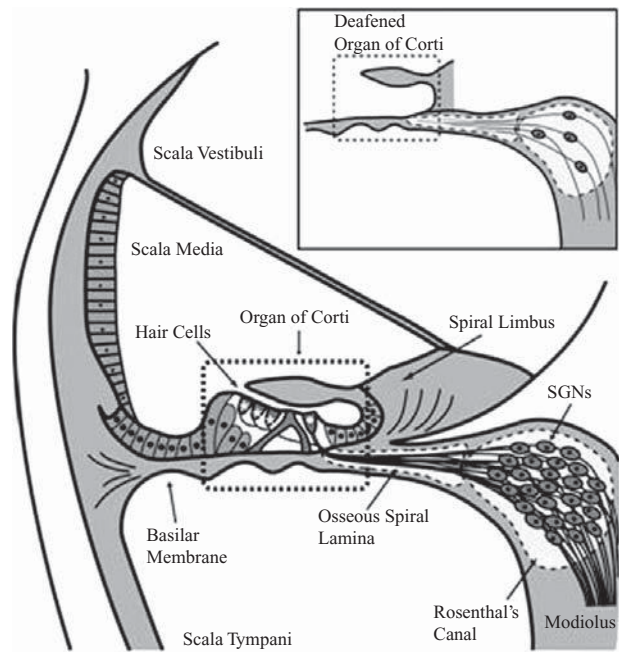


Figure 17-3. Schematic of the normal and degenerating cochlea. In a cross-section of the mammalian cochlea, inner (labeled with a white dot) and outer (highlighted with asterisks) hair cells in the organ of Corti are innervated by peripheral processes of spiral ganglion neurons (SGNs). Deafness induced by ototoxic drugs or noise results in death of these hair cells in the organ of Corti, leading to secondary degeneration and loss of SGNs (see inset). Adapted with permission from Hurley et al., 2007.

persist in adult SGNs, suggesting an ongoing physiologic role for neurotrophin signaling in adulthood (Figure 17-3).

4.2. Afferent activity from hair cells

In addition to trophic support, SGNs require afferent activity from sensory hair cells for survival. In the organ of Corti, inner hair cells stimulate SGNs by secreting glutamate, which activates two classes of receptors: the N-methyl-D-aspartate and α -amino-3-hydroxy-5-methylisoxazole-4-propionate receptors. These ligand-gated receptors are ion channels that open on activation, causing an influx of cations into the SGN. This gradually depolarizes the neuron, activating another class of voltage-gated ion channels – the L-type Ca^{2+} channels. Blockade of L-type Ca^{2+} channels with inhibitors (nifedipine and verapamil) abolishes the trophic effect of depolarization in SGN cultures, demonstrating that Ca^{2+} entry is necessary for SGN survival. Furthermore, both glutamate receptors and L-type Ca^{2+} channels are present in SGNs, suggesting that these ion channels mediate afferent activity initiated by hair cells.

A regulated influx of Ca^{2+} in neurons is essential to trigger intracellular survival signaling cascades linked to

SGN survival. Considering the diversity and cross-talk among signaling cascades, one approach to understanding SGN physiology has been to identify the downstream targets that promote survival and then examine the corresponding upstream signaling cascades. Degenerating SGNs show a decline in phosphorylation of the nuclear transcription factor, cyclic adenosine monophosphate response element binding protein (CREB). CREB is a downstream target that is indirectly activated by Ca^{2+} influx. CREB regulates the expression of many genes necessary for neuronal function and survival. Phosphorylation of CREB can be induced by at least two pathways: the Ras-MAPK pathway and the Ca^{2+} /calmodulin-dependent kinases, both of which are activated by Ca^{2+} influx. MAPK and Ca^{2+} /calmodulin-dependent kinases phosphorylate CREB at specific amino acid residues, enabling CREB to bind to distinct nucleotide sequences and promote transcription of selected genes such as BDNF. In primary cultures of postnatal SGNs, inhibitors of either MAPK or Ca^{2+} /calmodulin-dependent kinases reduce SGN survival, suggesting that these pathways are necessary for SGN survival. Because BDNF mRNA transcripts in SGNs are expressed in a manner that is dependent on neuronal activity, it remains likely that BDNF produced by SGNs can promote survival via an autocrine signaling loop involving their TrkB receptors. Another mechanism by which activity-induced depolarization can promote SGN survival is via phosphorylation (inactivation) of proapoptotic Bad by Ca^{2+} /calmodulin-dependent kinase II.

4.3. Molecular manifestations of spiral ganglion neuron death

The death of SGNs is not remarkably different from that of most other neurons, but we will highlight certain features that are manifested in degenerating SGNs. When aminoglycoside antibiotics are administered to rats to induce hair cell death, a dramatic reduction in TrkB expression in SGNs and their peripheral processes is observed. Concomitantly, increased expression of the p75 neurotrophic receptor (p75NTR) occurs in both SGNs and Schwann cells. Although classified as a neurotrophic receptor like TrkB and TrkC, p75NTR has diverse roles in the nervous system depending on the pathological status of the cells. In healthy cells, the p75NTR receptor can increase the affinity of binding between NGF and its cognate TrkA receptor, or BDNF and TrkB. However, under conditions of trauma and inflammation, p75NTR can trigger apoptosis.

The mechanisms underlying the proapoptotic activity of p75NTR are largely unknown. However, this

signaling may be related to whether the ligand is in a mature or immature form. In their mature forms, neurotrophins BDNF and NT3 support SGN survival. Like many hormones and enzymes, neurotrophins are produced initially as pro-neurotrophin forms consisting of a pro-domain linked to the mature neurotrophin. Proteolytic cleavage releases the mature neurotrophins, enabling them to mediate most of their biological functions, including survival. However, pro-neurotrophins can bind to p75NTR at subnanomolar concentrations to induce cell death, illustrating the dependence of signaling by neurotrophins on their state (whether pro- or mature neurotrophin). In rat cochleae exposed to aminoglycoside antibiotics, the accumulation of uncleaved pro-BDNF and the augmented expression of p75NTR in the cochleae suggest that this mechanism may contribute to SGN degeneration. Although it remains to be determined whether this mechanism contributes to SGN death in humans, new lines of evidence support this hypothesis. For example, pro-neurotrophin forms of NGF accumulate in brains of humans with Alzheimer's disease. Furthermore, pro-NGF isolated from these diseased brains rapidly triggers death in cultured neurons. It is unclear how p75NTR signals cell death, but an increase in JNK activity has been associated with p75NTR-dependent apoptosis. In addition, degenerating SGNs show increased phosphorylation of c-Jun, indicating activation of the JNK signaling pathway. Thus, similar to what is known about death of sensory hair cells, activation of the JNK signaling pathway may contribute to SGN apoptosis.

Hair cells do not always perish immediately after ototoxic drug exposure; they sometimes undergo a progressive atrophy with time. In particular, aminoglycoside antibiotics, which have a half-life in serum of approximately 3 to 5 hours, are not efficiently cleared from the inner ear, resulting in an extended half-life in inner ear tissues and fluids that may exceed 30 days. This protracted retention in the cochlea leads to hair cell death, followed by gradual death of SGNs. Apoptotic features can be observed within somas of degenerating SGNs weeks and months after the initial traumatic insult, in part because of the gradual nature of hair cell death. Some of the apoptotic features in degenerating SGNs include increased terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, cytochrome c release, activation of caspase-9, and increases in the caspase-cleaved fragment of poly (ADP-ribose) polymerase. These features suggest the involvement of members of the mitochondrial cell death pathway in regulating apoptosis in SGNs, and over-expression of Bcl-2 in

SGN cultures from postnatal or adult rats significantly increases SGN survival.

4.4. Therapeutic interventions to prevent SGN death

Over the past two decades, significant improvements have occurred in the development of hearing aids and cochlear implants to help hearing-impaired and deaf individuals overcome their disabilities. The main therapeutic effect of hearing aids is sound amplification, with the goal of delivering sound that is loud enough to be detected by remaining hair cells without being uncomfortably loud. In contrast, cochlear implants involve functional electrical stimulation of SGNs and thus do not require any hair cell function to be therapeutic. Cochlear implants are used successfully in many patients with severe hearing loss in which hearing aids are unlikely to provide therapeutic benefit. However, the success of cochlear implants depends in part on a viable population of SGNs. This has been an indication for commencing cochlear implant surgery sooner rather than later after the death of hair cells. Some studies have focused on strategies aimed at delivering pharmacological agents to target specific growth factor signaling pathways to boost SGN survival. Not surprisingly, delivery of BDNF, NT3, and GDNF using mini-osmotic pumps or viral vectors promotes SGN survival. Unfortunately, clinical applications of neurotrophins as part of a regimen to treat sensorineural hearing loss are complicated by the fact that SGNs degenerate rapidly when exogenous neurotrophins are withdrawn. In addition, peripheral processes in BDNF-treated SGNs show a disorganized sprouting pattern, reminiscent of abnormal branches and sprouting of gustatory fibers in tongue tissues of transgenic mice over-expressing BDNF. Whereas these observations pinpoint the potential dangers of ectopic neurotrophin delivery, they also illustrate the necessity of fully understanding the biological processes underlying neurotrophin action in relation to SGN survival. Such an understanding will inform therapeutic strategies aimed at complementing cochlear implants with a drug-based therapy.

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18

Cell Death in the Olfactory System

Pawel Kermer

1. INTRODUCTION

The olfactory system is one of the most plastic regions in the brain, where neurons are continuously replaced and neuronal circuits are modulated throughout life. Although there is a constant replacement of receptor neurons in the adult olfactory epithelium, there is a perpetual integration of new neurons into the adult olfactory bulb. Hence a fine-tuned program to balance neuronal apoptosis and survival is necessary. This chapter summarizes current knowledge about life and death in the adult olfactory epithelium and olfactory bulb after illustrating normal olfactory anatomy. Finally, olfactory neuronal death is revisited in the context of neurodegenerative diseases.

2. ANATOMICAL ASPECTS

The neural system for smell is composed of the olfactory epithelium in the nose, the fila olfactoria forming the first cranial nerve (olfactory nerve), the olfactory bulb and tract, and depending cortical areas (Figure 18-1). The olfactory pathway is unique because smell is the only sense that reaches the phylogenetic old cortical regions without being relayed through the thalamus. The olfactory epithelium covers an area of approximately 2 to 5 cm² in the dorsal posterior recess of the nasal cavity and contains receptor cells, basal cells, supporting cells, and glands (Figure 18-2). Odors are sensed by receptors on the endings of the short peripheral processes of bipolar receptor neurons. Their unmyelinated axons are bundled and surrounded by a Schwann cell, forming approximately 20 so-called fila olfactoria on each side, which together are considered as olfactory nerves. Olfactory receptor neurons (ORNs) project through the cribriform plate of the ethmoid bone

and terminate in the olfactory bulb (OB), a part of the telencephalon lying below the frontobasal cortex. Here, ORN axons form the first synapse of the olfactory pathway within specialized synaptic areas called glomeruli mainly on large mitral cells and on small tufted cells, the main output neurons of the OB. Sensory information is modulated in the OB by two types of inhibitory interneurons, granule cells and periglomerular cells, which receive synaptic input from other parts of the nervous system and to some extent also from ORNs (Figure 18-2). Neurites from mitral and tufted cells form the olfactory tract (second neuron), dividing into medial and lateral olfactory striae in front of the anterior perforated substance. Although the lateral stria projects to the amygdala and prepyriform area, where it connects to the third olfactory neuron, reaching cortical projection fields of the olfactory system in the entorhinal area and parahippocampal gyrus, fibers of the medial stria terminate in the septal area below the corpus callosum, from where the third neuron projects to the contralateral hemisphere via the anterior commissure. Other projections reach the habenular nucleus, reticular formation, and tegmental nuclei via the longitudinal striae, striae medullares, and medial forebrain bundle (Figure 18-1).

3. LIFE AND DEATH IN THE OLFACTORY SYSTEM

3.1. Olfactory epithelium

The olfactory epithelium was identified as an exceptional brain region with regenerative capacity and continuous turn-over of neuronal cells more than 30 years ago (Graziadei, 1973; Graziadei and Monti Graziadei, 1980). As holds true for large parts of the rest of the nervous system, the number of ORNs in the developing rat brain is fine-tuned predominantly by apoptosis

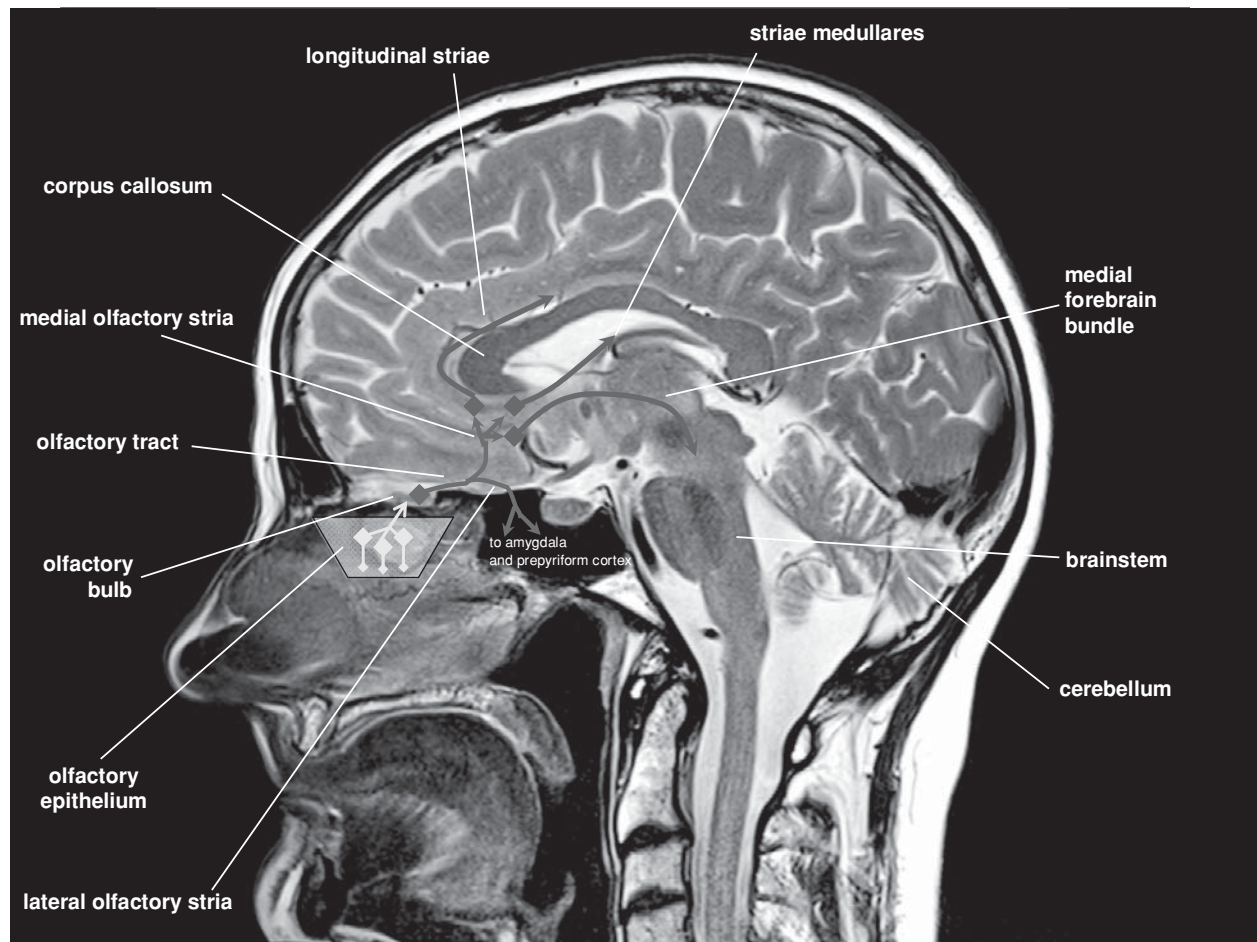


Figure 18-1. Gross anatomy. Parasagittal MRI scan of the human brain (courtesy of J. Buhk, Department of Neuroradiology, University Medical Center Göttingen) illustrating the olfactory pathway. Olfactory receptor neurons in the olfactory epithelium (green) constitute the olfactory nerve (first cranial nerve), with their axons projecting to the olfactory bulb forming synapses on the second olfactory neurons (red). Forming the olfactory tract, these neurons project through the lateral olfactory stria to amygdala and prepyriform cortex, where the information is relayed. Through the medial olfactory stria, neurons of the second olfactory neurons reach the septal area, where the information is transferred to the third olfactory neurons (blue), which in turn give rise to numerous projection to the contralateral hemisphere, the brainstem, and other regions (see text for details). See Color Plate 19.

(Figure 18-2), with a peak around E12 (Pellier and Astic, 1994) displaying typical morphological criteria under the electron microscope. However, unlike other parts of the nervous system, ORNs undergo both genesis and apoptosis throughout adult life. Initially, ORN apoptosis in intact olfactory epithelium was demonstrated by electron microscopy (Magrassi and Graziadei, 1995). Later, DNA fragmentation was documented histologically employing the terminal dUTP nick end labeling (TUNEL) technique in ORNs in the normal adult rat (Deckner et al., 1997) and mouse (Holcomb et al., 1995). Retroviral labeling of progenitor cells, the so-called globose basal cells in the olfactory epithelium (Suzuki and Takeda, 1991; Suzuki, 2004), revealed an ORN lifespan of approximately 1 month (Caggiano et al., 1994). However, some ORNs are believed to live more

than 3 months (Hinds et al., 1984; Mackay-Sim and Kittel, 1991x), depending on environmental input (see Farbman, 1990, for review). In aged mice, apoptosis is increased again when compared with normal or young animals with high expression levels of procaspase-3 and Bax (Robinson et al., 2002), suggesting increased fragility of the aged olfactory system.

The pathways underlying ORN apoptosis *in vivo* have mainly been studied by manipulating ORN death by lesion models such as naris occlusion, bulbectomy, and olfactory nerve transection. Although the latter two cause massive ORN apoptosis, sensory deprivation by naris occlusion leads to decreased ORN density and smaller size of the downstream regions in the olfactory bulb within 30 days (Farbman et al., 1988). Because the number of ORNs immunoreactive for olfactory marker

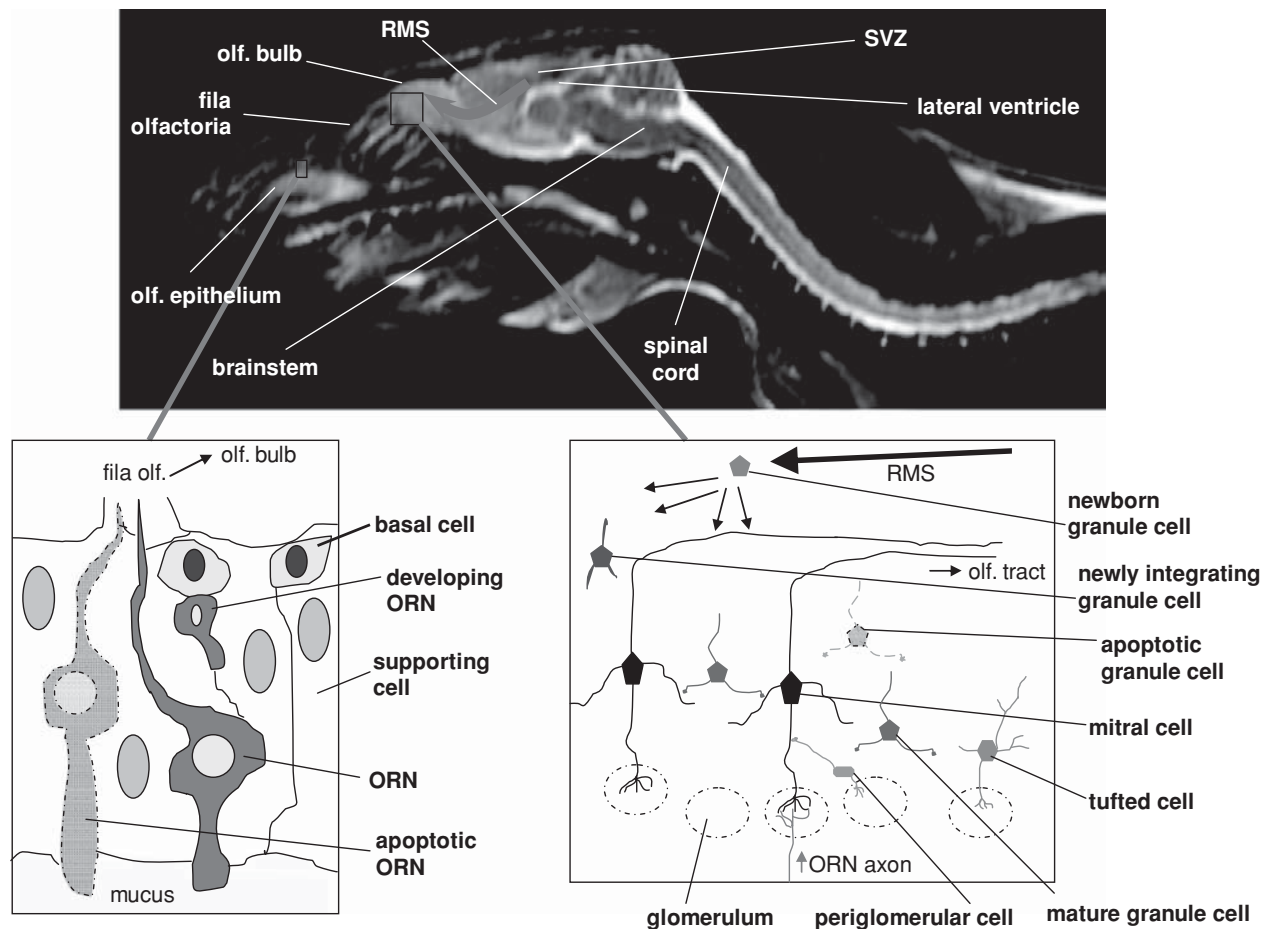


Figure 18-2. Olfactory life and death on a microscopic level. Parasagittal magnetic resonance imaging scan of the rat brain (courtesy of J. Buhk, Department of Neuroradiology, University Medical Center Göttingen) illustrating apoptotic ORN turn-over in the olfactory epithelium (lower left panel) and replacement by basal daughter cells. The lower right illustration shows olfactory bulb anatomy on the cellular level, with replacement of apoptotic granule cells by newly integrating granule cells that migrated through the rostral migratory stream (RMS) after they were born in the subventricular zone (SVZ) adjacent to the lateral ventricle. See Color Plate 20.

protein stays constant, reduced ORN numbers after naris occlusion are believed to be due to impaired neurogenesis (Cowan and Roskams, 2002). DNA laddering after olfactory nerve transection or bulbectomy peaks between 24 and 48 hours post-lesion (Cowan and Roskams, 2002; Suzuki, 2004). Although surgical removal of the olfactory bulb, in contrast to axotomy, allows replaced ORNs to grow axons toward the former region of the olfactory bulb, they are deprived from their target and die chronically. This is reflected by the fact that all ORNs on the bulbectomized side appear to have a substantially shortened lifespan of less than 14 days (Schwob et al., 1992), possibly because of a lack of target-derived trophic support.

On the molecular level, mRNAs for Fas, Fas ligand, tumor necrosis factor receptor 1, and its ligand, tumor necrosis factor alpha (TNF- α), have been detected in the olfactory epithelium of adult rats (Farbman et al., 1999),

suggesting an important role of the so-called extrinsic apoptosis pathway for ORN apoptosis. Indeed, addition of Fas ligand or TNF- α to organotypic cultures of fetal E19 rat olfactory epithelium causes an increased apoptosis after 4 to 6 hours (Cowan and Roskams, 2002). There is more evidence, however, for the relevance of the mitochondria-dependent intrinsic apoptosis pathway because ORN death can be modulated by pro- and antiapoptotic members of the Bcl protein family. For example, over-expression of the antiapoptotic Bcl-2 protein in transgenic mice protects ORNs from apoptosis induced by bulbectomy for at least 5 days (Jourdan et al., 1998), a time point at which lesion-induced ORN loss is believed to be over. In line with that, knock-out mice for the proapoptotic Bax protein are protected from bulbectomy-induced ORN loss (Robinson et al., 2003), even though neither expression of Bcl-2 nor Bax could be located in ORNs so far. Further support

for the involvement of the intrinsic apoptosis pathway in ORN loss comes from data by Cowan and co-workers demonstrating caspase-9 and -3 as major drivers for ORN apoptosis, both during development and in the mature brain (Cowan et al., 2001; Cowan and Roskams, 2004). Both caspases are present in mature ORNs, and their expression levels increase within hours upon apoptosis initiation by bulboectomy. Caspase-3 seems first to be activated at the synapse proceeding through the axon to the cell body, suggesting a timed activation pattern within the ORN population. Underscoring the importance for caspase-3 as executioner of ORN apoptosis also during development, caspase-3 knockout mice display an expanded ORN population (Cowan et al., 2001). On the other hand, not only caspase-3 mRNA, but also mRNAs for caspase-1 and -2 have been detected in the olfactory epithelium of fetal and adult rats (Suzuki and Farbman, 2000). In the case of caspase-2, mRNA is not detectable between 3 and 5 days after bulboectomy but reappears within 21 days, suggesting that caspase-2 may be expressed in ORNs and contribute to the propagation of cell death. More evidence for caspase-dependent ORN apoptosis comes from studies employing caspase inhibitors, which not only blocked apoptosis in organotypic cultures of the olfactory epithelium after treatment with TNF- α , but also reduced apoptosis under control conditions (Suzuki and Farbman, 2000).

Finally, ORNs contain several caspase substrates that eventually result in DNA fragmentation. However, their significance for ORN remains unresolved. Cleavage of alternative caspase targets, like amyloid precursor-like protein 2, which is presumed to mediate axonal outgrowth in ORNs, reflects the spatial caspase activation, further supporting the relevance of caspases for ORN loss in normal and lesioned olfactory epithelium (Cowan and Roskams, 2002).

3.2. Olfactory bulb

Besides the hippocampus, the olfactory bulb is the only region in the adult mammalian brain where the integration of newly generated neurons into pre-established neuronal circuits is uncontroversial (Altman, 1969; Gage, 2002; Hack et al., 2005; Gould, 2007). Neuronal progenitors destined for the olfactory bulb are generated in the subventricular zone (SVZ) between the lateral ventricle and the striatum from astrocytic neural stem cells (Doetsch et al., 1999; Garcia et al., 2004). Although evidence for its existence is lacking in humans (Sanai et al., 2004), in the rodent, newly produced postmitotic neuroblasts migrate tangentially along the so-called

rostral migratory stream (RMS) (Figure 18-2), where they are shielded against surrounding brain tissue by glial cells forming a tube-like structure (Lois et al., 1996; Anton et al., 2004). More than 30,000 neuroblasts exit the rodent SVZ each day (Alvarez-Buylla et al., 2001), but only a very limited number is integrated into the olfactory bulb after radial invasion from the migratory path (Lledo and Saghatelian, 2005). Newborn olfactory neurons can only mature into two types of inhibitory interneurons. The vast majority differentiate into GABAergic granule cells (Figure 18-2), whereas only very few give rise to periglomerular cells expressing GABA and/or the dopamine-synthesizing enzyme tyrosine hydroxylase (Lledo and Saghatelian, 2005). Both types of neurons only form local synapses and probably can directly or indirectly modulate the processing of sensory input by mitral and tufted cells, the olfactory bulb output neurons (Lledo et al., 2006). Surprisingly, only approximately 50% of the newly generated interneurons survive more than a month (Petreanu and Alvarez-Buylla, 2002; Winner et al., 2002) before they die by apoptosis (Figure 18-2), as revealed by TUNEL labeling (Winner et al., 2002). This waste of resources poses the question regarding the functional relevance of adult neurogenesis. Although the answer still remains obscure, Lledo and co-workers (2006) offered a scheme of newborn neurons functioning at the cellular and network levels, either leaving existing neurons unchanged, changing existing networks, or just replacing apoptotic cells displaying adaptable or prespecified functions. Hence the hypothesis that bulbar neurogenesis mediates the adjustments of sensory processing in response to the constantly changing olfactory input appears feasible.

Be that as it may, a clear correlation between the survival of newborn bulbar granule cells and olfactory input has been demonstrated (Corotto et al., 1994; Rochefort et al., 2002; Miwa and Storm, 2005; Saghatelian et al., 2005). On the molecular level, various factors have been shown to regulate neuronal survival in the olfactory bulb. For example, glutamate, the main excitatory amino acid in the brain, has been shown to enhance survival of SVZ-derived neurons in a dose-dependent manner, whereas phosphatase and tensin homolog, a lipid phosphatase with proapoptotic activity, has been identified as negative regulator (Brazel et al., 2005; Li et al., 2002). Moreover, loss or inactivation of the polysialylated isoforms of neural cell adhesion molecule (NCAM) leads to reduced survival of postnatally generated immature neurons in the olfactory bulb in response to neurotrophic factors due to an upregulation of the p75 neurotrophin death receptor (Gascon et al., 2007). In line with the increased death of olfactory bulb neurons in

the absence of NCAM, NCAM^{-/-} mice display an approximately 30% reduction in olfactory bulb size and an approximately 10% reduction in overall brain size (Cremmer et al., 1994; Tomasiewicz et al., 1993; Gheusi et al., 2000). Furthermore, extracellular signal-regulated kinase 1/2 (Erk1/2) as part of the mitogen-activated protein kinase (MAPK) pathway have been identified as promoters of granule cell survival in the olfactory bulb. Odor exposure of mice *in vivo* resulted in MAPK activation in granule cells within 10 minutes, with an increased survival of newly formed granule cells (Miwa and Storm, 2005). In contrast to ORNs, where expression of the antiapoptotic Bcl-2 and the proapoptotic Bax protein could not be demonstrated so far, increased Erk phosphorylation after odor exposure culminated in increased expression levels of the antiapoptotic Bcl-2 protein in the olfactory bulb (Miwa and Storm, 2005). Similarly, adult Bax-deficient mice exhibited markedly reduced apoptosis in the olfactory bulb, as revealed by TUNEL staining in comparison with wild-type mice (Kim et al., 2007). At the same time, neurons expressing active caspase-3, which are physiologic in wild-type mice, could not be detected in Bax knockout mice. Interestingly, lack of apoptosis in the olfactory bulb of Bax-deficient mice was neither associated with increased size of the olfactory bulb nor changes in the number of proliferating neuroblasts in the SVZ, but rather caused an accumulation of ectopic neurons in the RMS as a result of an impairment of RMS glial tube formation (Kim et al., 2007). Conversely to the hypothesis suggesting a relevance of granule cell turn-over in the olfactory bulb for processing of the sensory information (Lledo et al., 2006), and in conflict with the correlation between the survival of newborn bulbar granule cells and olfactory input (Corotto et al., 1994; Rochefort et al., 2002; Miwa and Storm, 2005; Saghatelian et al., 2005), adult and aged Bax knockout mice show normal olfactory behavior and odor discrimination (Kim et al., 2007). Together, these most recent data question the requirement of granule cell apoptosis in the olfactory bulb for odor discrimination or odor memory.

4. OLFACTION IN AGING AND NEURODEGENERATIVE DISEASE

Impairment of smell perception with age in humans was discovered more than 20 years ago (Doty et al., 1984; Stevens and Cain, 1987) and was believed to be a matter of senescence. New data, however, indicate that a significant decline in odor discrimination ability can already be documented in the second half of the fourth decade in human life (Hawkes, 2006). Interestingly, the sensation of high hedonic or low-intensity odors is more severely

affected than the sensation of unpleasant ones (Hawkes, 2006). In rodents, this decline in olfactory discrimination ability correlates with a profound decrease in SVZ neurogenesis rather than increased apoptosis in the olfactory bulb. Still, decreased neurogenesis results in a reduction in the number of new neurons in the olfactory bulb affecting both periglomerular and granule interneurons (Enwere et al., 2004).

The discovery of hyp- or anosmia as common feature in idiopathic Parkinson's disease (PD) and dementia of the Alzheimer type (AD) has boosted the interest in olfactory dysfunction in neurodegenerative diseases. In AD, typical histological abnormalities throughout the olfactory system are well documented (Yamagishi et al., 1994; Braak and Braak, 1998; Kovacs et al., 2001), and it has been suggested that AD could be diagnosed by biopsy of the nasal olfactory epithelium (Talamo et al., 1989). However, this procedure has not entered the clinic for several reasons, including doubts on specificity, histological limitations, and the fact that there is a constant replacement of ORNs that might be even accelerated in AD (Hawkes, 2006). Nevertheless, hyposmia/anosmia has been identified as risk factor for subsequent cognitive failure and AD, especially in combination with specific genetic markers (see Graves et al., 1999; Hawkes, 2006, for review).

Olfactory dysfunction in PD was first reported more than 30 years ago (Ansari and Johnson, 1975), and loss of smell was suggested as risk factor, as well as a screening tool for the development of PD in recent prospective studies (Sommer et al., 2004; Ross et al., 2006; Haehner et al., 2007). This is also of clinical importance with regard to differential diagnosis of PD because there are other neurodegenerative diseases associated with PD symptoms in which loss of smell is absent or much less severe (e.g., multisystem atrophy, essential tremor and others; for review see Hawkes, 2006). Pathological hallmarks of PD (Lewy neuritis and Lewy bodies) are first found in the brainstem, olfactory bulb, and associated anterior olfactory nucleus in the olfactory tract long before the disease becomes clinically apparent (Braak et al., 2003), with Lewy bodies being found in mitral cells of the olfactory bulb (Daniel and Hawkes, 1992) and dystrophic neurites being present in the olfactory epithelium (Crino et al., 1995). Although a detailed analysis of apoptosis in the olfactory bulb beyond anatomical description is still lacking, significant cell loss in the PD olfactory bulb is beyond doubt (Hawkes, 2006). From results in the rodent, it appears that this cell loss can at least partially and temporarily be compensated by increased neurogenesis and cell replacement in the olfactory bulb (Yamada et al., 2004), thereby slowing down symptom onset.

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Contribution of Apoptosis to Physiologic Remodeling of the Endocrine Pancreas and Pathophysiology of Diabetes

Nika N. Danial

1. INTRODUCTION

Homeostatic control of blood glucose levels is critically dependent on the balance of glucagon and insulin, two counteracting pancreatic hormones secreted by endocrine cells within the islets of Langerhans – alpha and beta cells, respectively. Elegant biochemical studies combined with metabolic flux analysis uncovered the unique ability of beta cells to sense blood glucose fluctuations and to fine tune insulin secretion accordingly.¹ A high-capacity glucose transport system, a low-K_m glucose phosphorylating activity catalyzed by glucokinase (GK), and the ability to channel the majority of glycolytically derived pyruvate to the mitochondrial tricarboxylic acid (TCA) cycle constitute essential metabolic design features that endow beta cells with a specialized secretory function.² The increase in intracellular adenosine triphosphate (ATP)/adenosine diphosphate (ADP) ratio on mitochondrial metabolism of nutrients is among the metabolic coupling factors connecting fuel oxidation to insulin secretion. A rise in ATP/ADP ratio in turn leads to closure of ATP-sensitive K (K_{ATP}) channels at the plasma membrane, followed by membrane depolarization and influx of Ca²⁺ necessary for release of insulin granules.³ The two aspects of beta cell biology that contribute significantly to euglycemia are glucose dose responsiveness of insulin secretion and the remarkable plasticity of beta cell mass to meet insulin demand under physiologic and pathophysiologic nutrient stress.^{4,5} Beta cell mass is the net outcome of neogenesis (formation of new beta cells from non-beta cell precursors), replication of preexisting beta cells, beta cell size, and apoptosis.⁶ The integration of beta cell function and mass is further ensured through nutrient sensing pathways that concomitantly signal insulin secretion and modulate beta cell replication and survival.^{7,8} This chapter highlights the

apoptotic mechanisms operative in beta cells that influence the dynamic control of beta cell mass during the developmental remodeling of the endocrine pancreas and in the pathogenesis of diabetes.

2. APOPTOSIS IN PHYSIOLOGIC CONTROL OF BETA CELL MASS

The transition from fetal to adult beta cell during physiologic remodeling of endocrine pancreas is heavily influenced by apoptosis. During fetal life in both rodents and humans, large and rapid expansion of beta cell mass is marked mainly by beta cell neogenesis, endowing the fetal pancreas with a suitable number of beta cells.^{9,10} Although beta cell mass continues to expand in neonates, the net beta cell mass remains unchanged because of a concomitant wave of apoptosis, which in rodents, occurs around the weaning period, and in humans, around the time of birth.^{11,12} Neonatal beta cell apoptosis is part of a physiologic program to remodel the endocrine pancreas^{4,12} and may further serve as a quality-control mechanism to select for a functional pool of beta cells with appropriate insulin secretion response to glucose.^{13,14,15} Persistent beta cell proliferation and apoptosis beyond the neonatal phase of pancreatic development was recently found in biopsies of newborns and children with hyperinsulinism and hypoglycemia of infancy,¹¹ a metabolic disorder in which the lack of proper neonatal beta cell mass remodeling interferes with the fetal to adult beta cell transition, leading to retention of fetal beta cells incapable of eliciting an insulin secretory response that matches the level of glycemia.¹⁶ The molecular mechanisms controlling the change in apoptotic rate during fetal to neonatal beta cell transition are not fully understood. Direct correlations exist between the wave of apoptosis and expression

pattern of multiple cell death/survival molecules in the developing pancreas. Notably, the antiapoptotic BCL-2 protein and the inhibitor of apoptosis protein survivin, which inhibits caspase-3 and -9,¹⁷ display high expression levels in fetal beta cells but are undetectable in the postnatal period.^{18,19} Furthermore, the neonatal wave of apoptosis is marked by high expression of the dephosphorylated, apoptotically active form of the BCL-2 family protein BAD.^{20,21,22} This is consistent with the observation that insulin-like growth factor (IGF) II, a known beta cell survival factor that targets BAD phosphorylation²³ and stimulates beta cell survival,²⁴ declines during neonatal pancreatic development.^{25,26,27} Of note, low levels of fetal IGF-II levels are associated with elevated beta cell apoptosis and gestational diabetes due to maternal diet low in proteins.²⁸

In the adult, beta cell mass is linearly proportional to body weight²⁹ and is under tight homeostatic control so that insulin secretion is proportional to insulin demand. Apoptosis influences the beta cell mass dynamics in the adult. For example, a 2.5-fold increase in beta cell mass during pregnancy^{30,31} is followed by rapid and efficient involution of beta cell mass postpartum because of a significant increase in apoptosis.³² Similarly, increase in beta cell function^{33,34} and mass^{35,36,37,38} during weight gain associated with obesity is an adaptive response to compensate for obesity-induced insulin resistance, a state in which liver and peripheral tissues such as muscle and fat become less sensitive to the action of this hormone and more insulin is needed to preserve euglycemia.^{39,40} In addition to increased proliferation and beta cell hypertrophy, attenuated apoptosis contributes to obesity-induced beta cell mass expansion.^{41,42,43} Studies are just beginning to unravel the functional relevance of the intrinsic and extrinsic pathways of apoptosis in beta cell mass adaptation. As such, genetic models have provided evidence that BAD^{36,44} and caspase-8⁴⁵ are but two apoptotic modulators of obesity-associated beta cell mass expansion. The importance of this homeostatic response of beta cell mass to insulin resistance is underscored by the fact that its insufficiency is associated with type 2 diabetes^{46,47,48,49} as detailed in the following sections.

3. CONTRIBUTION OF APOPTOSIS TO BETA CELL MASS INADEQUACIES IN DIABETES

Loss of functional beta cell mass is central to the etiology of both type 1 and type 2 diabetes.^{35,50,51,52} Although the distinction between these two disease subtypes is based on differences in the nature of beta cell failure, the time of disease onset, and genetic predisposition,

accumulating evidence suggests that the common features between these two subtypes are more numerous than previously anticipated. Indeed, beta cell apoptosis is a common end point in both subtypes, which is further influenced by genetic and environmental factors. In type 1 diabetes (T1D), absolute insulin deficiency is due to the autoimmune-mediated loss of beta cells and the associated inflammatory immune response.⁵² In type 2 diabetes (T2D), beta cell apoptosis is induced by metabolic stress that accompanies chronic exposure to elevated levels of glucose and saturated fatty acids, oxidative stress, and endoplasmic reticulum (ER) stress because of accumulation of unfolded proteins.^{8,53,54,55} Recent studies also show that the damage inflicted by inflammatory cytokines is not unique to T1D; rather the metabolic stress imposed on beta cells in T2D leads to their secretion of inflammatory cytokines and subsequent cytotoxicity.⁵⁶ The following sections review the beta cell defect and apoptotic stimuli in each diabetes subtype.

3.1. Beta cell death in the development of T1D

T1D is marked by autoimmune destruction of islet beta cells by cytotoxic T-lymphocytes (CTLs) that recognize beta cell-derived self-antigens. Progression of the type 1 disease follows a series of histopathologically defined stages that includes infiltration of lymphocytes in the area surrounding the islets of Langerhans, also known as insulinitis, followed by production of inflammatory cytokines and fulminating immune attack.⁵² Permanent loss of functional beta cell mass is accompanied by blunted insulin secretion, glucose intolerance, and insulin dependency for life. Although characterization of the type 1 disease in humans has been limited to *in vitro* islet cultures and gene expression profiling of different disease stages, mouse models of the disease, especially the nonobese diabetic (NOD) mouse, as well as multiple T cell receptor TCR transgenic lines, have proved instructive in uncovering some of the molecular mechanisms underlying the breakdown of immune barrier and the cellular and molecular components of beta cell destruction.⁵⁷ However, despite multiple common elements in disease progression, the extent to which modeling of the type 1 disease in rodents phenocopies that in humans is not fully understood.

Autoantigens in T1D consist of beta cell-derived peptides that are most likely encountered during the wave of neonatal beta cell apoptosis.^{58,59,60} Antigen presenting cells (APCs) such as macrophages and dendritic cells engulf apoptotic beta cells and process and present beta cell-derived peptides that include, but may not

be limited to, peptides from insulin/proinsulin, glutamic acid decarboxylase, and the tyrosine phosphatase-like protein IA-2.⁶¹ Naïve beta cell-specific autoreactive CD4⁺ T cells that have escaped thymic negative selection become activated on encountering their cognate antigens presented by major histocompatibility complex (MHC) molecules on the surface of APCs in the pancreatic lymph nodes. On initial activation, CD4⁺ T cells migrate through the pancreas, where they reencounter their antigen, become activated, and remain in the islets (insulitis). Activated CD4⁺ T cells produce interleukin (IL)-2 and interferon (IFN)- γ , further stimulating APCs to secrete nitric oxide (NO), IL-1 β , and tumor necrosis factor (TNF)- α (Figure 19-1). TNF- α and IFN- γ stimulate secretion of chemokines from resident macrophages and endothelial cells, which help recruit CD8⁺ T cells.⁶² The latter secrete granules that contain perforin and the proapoptotic serine protease granzyme B. Perforin is a pore-forming protein⁶³ that on release generates membrane-penetrating tubular structures that allow the delivery of granzyme B to beta cells⁶⁴ (Figure 19-1). Granzyme B substrates include BID and caspase-3 and -7. Furthermore, CD8⁺ T cells trigger the extrinsic pathway of apoptosis by engaging FAS on the surface of beta cells.

Evasion of peripheral tolerance in T1D is influenced by genetic predisposition and environmental factors. For example, genome association studies have identified several candidate loci for T1D, among which are the HLA locus encoding MHC class I and II molecules required for antigen presentation and components of immune signaling pathways.^{65,66,67} This suggests that genetic predisposition may cause an exaggerated immune response, increasing the probability of autoreactive T-cell activation. In addition to genetic predisposition, environmental factors such as viral infection and toxins may also contribute to the activation of beta cell-reactive T cells.⁶⁸ For example, virally infected beta cells may produce cytokine/chemokines that help recruit and activate lymphocytes.⁶⁹ Furthermore, exposure of viral antigens on the surface of infected beta cells may activate autoreactive T cells by molecular mimicry of beta cell antigens.⁷⁰

3.2. Mechanisms of beta cell death in type 1 diabetes

Within the inflammatory cytokine milieu during the progression of T1D, the destruction of beta cells is believed to be apoptotic in nature.^{71,72,73} Beyond the autoimmune-mediated damage, beta cells also participate in their own demise by secreting inflammatory cytokines, chemokines, and NO.^{74,75} Loss-of-function

studies in mouse models of the disease have further revealed that interfering with the FAS/FASL, perforin/granzyme B, and signal transduction downstream of the inflammatory cytokines confers protection from T1D.^{76,77,78,79,80,81,82} However, protection is chiefly partial, indicating that these mechanisms work in concert. An overview of apoptotic pathways implicated in T1D is provided in the following sections.

3.2.1. Apoptosis signaling pathways downstream of death receptors and inflammatory cytokines

Direct contact of beta cells and T lymphocytes leads to activation of the extrinsic apoptotic pathway downstream of FAS and TNF receptor (TNFR), ultimately culminating in activation of caspase-8 and -10.⁸³ The subsequent transduction of the apoptotic signal is governed by protein-protein interaction domains that help assemble death-inducing signaling complexes (DISCs) on the cytoplasmic site of the death receptors⁸⁴ (Figure 19-1). Within the DISCs, receptors, adaptors, and pro-caspase-8 or -10 are assembled through selective homotypic engagement of protein-protein domains. These include the death domain (DD), the death effector domain (DED), and the caspase activation and recruitment domain (CARD) found in pro-caspases.⁸⁵ Structural studies have revealed that the three-dimensional structure of these protein interaction domains are remarkably similar.⁸⁶ The adaptor components of the DISCs vary depending on the death receptor. For example, in the case of FAS, the DISC is composed of FAS-associated DD-containing adaptor (FADD/MORT1) that engages the receptor through its DD and recruits pro-caspase-8 or -10 through its DED domain (Figure 19-1). On the other hand, TNFR-associated DD-containing adaptor TRADD lacks DEDs and recruits pro-caspases indirectly through FADD (Figure 19-1). The DISCs serve as molecular platforms for caspase activation and subsequent activation of effector caspases-3, -6, and -7.^{87,88} In addition to caspase-driven proteolytic destruction, a mitochondrial amplification loop is also operative in beta cells whereby the proapoptotic molecule BID is cleaved by caspase-8 to initiate a program of mitochondrial dysfunction, release of cytochrome c, and further activation of the caspase cascade.^{89,90} (Figure 19-1). Consistent with these observations, BID-deficient beta cells are protected from FAS- and TNF- α -induced apoptosis.⁸⁹ Furthermore, over-expression of BCL-2 protects human beta cells from apoptosis induced by inflammatory cytokines.⁹¹

Interference with FAS and TNFR signaling in certain settings protects against T1D. For example, mice

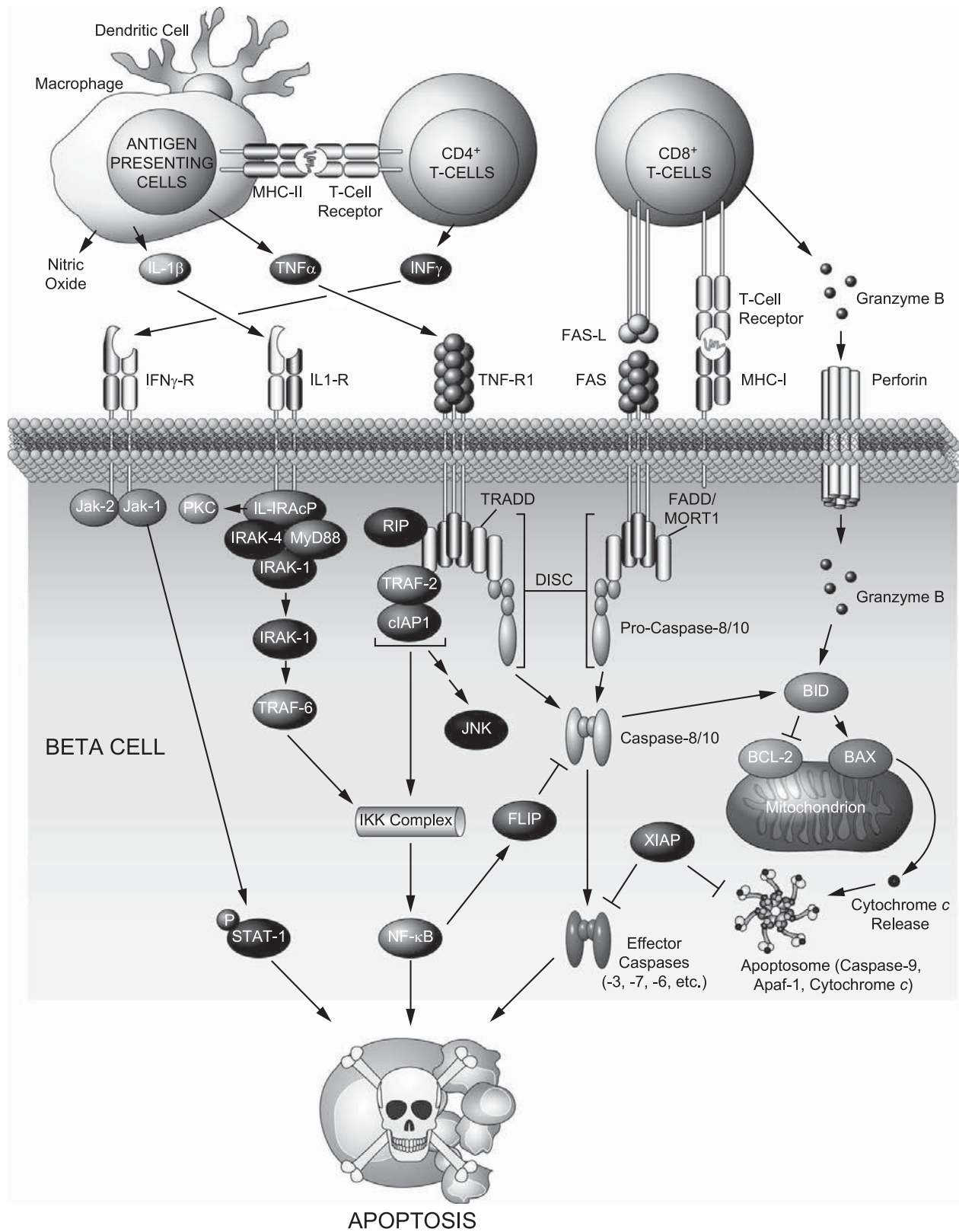


Figure 19-1. Signaling pathways leading to beta cell destruction in type 1 diabetes. Image courtesy of Eric Smith of Dana-Farber Cancer Institute. See text for details. See Color Plate 21.

over-expressing the dominant-negative form of FADD that binds the receptor but lacks DED⁷⁶ or transgenic animals expressing the soluble TNF decoy receptor lacking a transmembrane domain⁹² are more resistant to T1D. The efficiency of such genetic maneuvers may further lie on the simultaneous inhibition of FAS and TNFR, given that they share signaling intermediates such as FADD. This is especially relevant as beta cell-specific deletion of FAS alone was not sufficient to block disease progression.⁹³ Other strategies to preserve beta cell mass by blocking death receptor signaling include FLIP (FLICE/caspase-8 inhibitory protein)^{94,95,96} (Figure 19-1) and over-expression of the serine protease inhibitor CrmA, which inhibits caspase-8 and -10.⁹⁷

Accumulating evidence has recently suggested that components of death receptor signaling may have a non-apoptotic role beneficial to beta cell mass. These functions may in turn be developmentally regulated and/or dictated by distinct signaling complexes. For example, caspase-8 is required for physiologic beta cell growth⁴⁵ and glucose-stimulated insulin secretion.⁹⁸ Furthermore, differential recruitment of FLIP to caspase-8 at the cytoplasmic tail of FAS or selective association of DISC components in complexes devoid of FAS may carry non-apoptotic roles, including proliferation.^{98,99} Likewise, signaling downstream of TNFR1 can trigger apoptosis or proliferation, depending on the composition of complexes containing DD and DED proteins.¹⁰⁰ In addition to DISC, TNF- α can induce the assembly of a complex that contains TRADD, TNFR-associated protein-2 (TRAF-2), receptor-associated kinase RIP, and cellular inhibitor of apoptosis proteins cIAP1 and cIAP2,^{101,102} which enables activation of nuclear factor kappa B (NF- κ B).^{103,104} Depending on the cellular context, signaling downstream of NF- κ B may impart pro- or antiapoptotic signals. For example, FLIP is an NF- κ B target gene that inactivates the apoptosis arm of TNF- α signaling by inhibiting caspase-8.^{105,106} However, under other settings, NF- κ B activation is chiefly a proapoptotic signal in beta cells.¹⁰⁷ In light of these observations, any strategy devised to target death receptor signaling as a therapeutic approach in beta cell mass preservation must be selective for the apoptotic arm of the signaling cascade without interfering with proliferative signals emanating from the same receptor.

The effect of IL-1 β on beta cell survival/death depends on dose and duration of exposure. Acute exposure to IL-1 β stimulates insulin secretion¹⁰⁸ and beta cell proliferation,¹⁰⁹ allowing beta cells to compensate for insulin demand during inflammatory stress. However, in T1D, hyperglycemia prompts beta cells to secrete more IL-1 β .¹¹⁰ Chronic activation of signals emanating

from the IL-1 receptor (IL-1R) interferes with mitochondrial metabolism,¹¹¹ attenuates insulin secretion,¹¹² and induces apoptosis.¹¹³ In this context, blocking IL-1 β protects beta cells from apoptosis^{114,115} and ablation of IL-1R preserves beta cell mass in experimental models of T1D,⁸¹ either through immunomodulation and/or direct effects on beta cells. Beta cells express a low-affinity IL-1R1 that transduces the IL-1 β signal and a high-affinity IL-1R2 that serves as a decoy receptor.¹¹⁶ On ligand binding, a multiprotein complex assembles at the IL-1R that is composed of an accessory protein (IL-1RAcP) and two serine/threonine kinase IRAK-1 and -4, which are recruited to the receptor by the myeloid differentiation factor (MyD88)¹¹⁷ (Figure 19-1). Within this signaling complex, IRAK-4 phosphorylates IRAK-1, which then dissociates from the complex and binds TRAF6,¹¹⁸ leading to activation of IKK-NF- κ B signaling axes.¹¹⁹ Upregulation of several NF- κ B target genes such as iNos and Fas and downregulation of Bcl-2 compromises beta cell survival downstream of IL-1 β signaling.^{75,120} Consistent with these observations, blocking NF- κ B activation by transgenic expression of a nondegradable form of κ B α (the NF- κ B super repressor) preserves beta cell mass and protects against T1D.¹²¹

IFN- γ often acts synergistically with IL-1 β and TNF- α in apoptotic demise of beta cells.¹²² On engagement of its cognate receptor, IFN- γ activates the receptor-associated Janus kinase (JAK)-1 and -2, which subsequently phosphorylate select tyrosine residues on the cytoplasmic tail of the IFN- γ receptor (Figure 19-1). The signal transducer and activator of transcription (STAT)-1 docks on the phosphorylated receptor, becomes itself target of tyrosine phosphorylation, and dimerizes to translocate to the nucleus¹²³ (Figure 19-1). This signaling pathway is under negative regulation by inhibitors of cytokine signaling such as suppressors of cytokine signaling (SOCS)-1 and -3, which dock to the receptor, blocking activation of JAKs and access of STAT-1 to the receptor.^{124,125} The effect of IFN- γ on beta cell death is mediated through STAT-1¹²⁶; however, the precise nature of proapoptotic genes regulated by STAT-1 in these cells is not known.¹²⁰ Loss of STAT-1 function or gain of SOCS-1/-3^{127,128,129,130,131} function in beta cells preserves their viability in the presence of inflammatory cytokines and protects against T1D. The benefits of inhibiting JAK-STAT signaling in this case are two-fold: direct preservation of beta cell survival and immunomodulation.

3.2.2. Oxidative stress

Chronic intra-islet inflammation during progression of T1D can also activate the intrinsic pathway of

apoptosis through induction of oxidative stress and bioenergetic decline marked by attenuation of intracellular ATP^{132,133} and NAD⁺^{134,135,136} levels, massive increase in NO,⁷⁴ and elevation of cytoplasmic free Ca²⁺.¹³⁷ In certain settings, mitochondria dysfunction associated with these insults may also manifest in beta cell necrosis. NO is the major source of oxidative stress in beta cells, to which they are especially sensitive as a result of their low levels of antioxidant defense mechanisms.^{138,139,140} Although small amounts of NO are protective,^{141,142} supraphysiologic levels of this reactive oxygen species become cytotoxic during the course of T1D progression. Indeed, prevention of oxidative damage maintains beta cell viability in the presence of inflammatory cytokines *in vitro*^{133,143,144} and in experimental models of T1D *in vivo*.^{145,146,147,148,149,150,151,152,153}

In addition to macrophage production of NO, beta cells synthesize their own NO on cytokine-induced upregulation of inducible nitric oxide synthase (iNOS).⁷⁴ Accordingly, beta cells deficient in iNOS are more resistant to apoptosis induced by inflammatory cytokines,¹⁵⁴ and inhibition of iNOS function *in vivo* either through genetic approaches or pharmacological inhibitors is protective in preclinical models of T1D.^{145,155} Multiple intracellular targets of NO compromise both insulin secretion and viability of beta cells. NO-induced DNA damage activates poly (ADP-ribose) polymerase (PARP), a nuclear enzyme that is activated in response to DNA strand breaks, which in the process of DNA repair, consumes NAD⁺ and thereby depletes beta cell ATP levels.¹⁵⁶ Interestingly, toxins that cause T1D-like disease by destroying beta cell mass, such as streptozotocin and alloxan, are known to specifically deplete beta cells from their ATP and NAD⁺ reservoirs.¹⁵⁷ PARP-deficient mice are resistant to T1D,^{158,159,160} and PARP inhibitors are being pursued in antidiabetes strategies.¹⁵⁶ NO can also interfere with mitochondrial function at multiple levels, including inactivation of the mitochondrial TCA cycle enzyme aconitase, leading to diminished glucose oxidation and ATP synthesis^{161,162} and induction of mutations in mitochondrial genes such as components of respiratory chain complexes.¹⁶³

3.3. Mechanisms of beta cell death in type 2 diabetes

Increase in beta cell mass and function normally compensates for insulin resistance. Although obesity is associated with insulin resistance, not all obese individuals are diabetic as a result of sufficient beta cell compensation both at the level of function (insulin secretion) and beta cell mass expansion.^{33,34,38} In lean or obese

individuals, insulin resistance progresses to T2D on failure of beta cell mass expansion that may in turn be further exasperated by genetic and/or environmental factors.^{164,165,166} Beta cell failure is believed to occur early during the course of disease progression.¹⁶⁷ Thus T2D is marked by abnormalities in both insulin production and action. Although subject to some controversies in the past, the significance of beta cell demise in pathophysiology of T2D has increasingly gained support from analysis of human autopsies and rodent models of the disease.^{10,35,37} Indeed, assessment of a large number of pancreatic biopsies obtained from T2D subjects compared with lean and obese counterparts revealed 41% and 63% reduction in beta cell mass in lean and obese T2D individuals, respectively.³⁵ Remarkably, comparison of beta cell apoptotic, replicative, and neogenic rates indicated significant increase in apoptosis as the underlying mechanism of beta cell loss. Pathways implicated in beta cell apoptosis include glucolipotoxicity, oxidative stress, inflammation, and ER stress. Glucolipotoxicity, inflammation, and possibly ER stress may be shared apoptotic mechanisms in both disease subtypes, with differential predominance.

3.3.1. Glucolipotoxicity

Although elevated lipids signal beta cell mass expansion as an adaptive response to insulin demand (lipoadaptation),¹⁶⁸ chronic exposure to free fatty acids in the presence of elevated glucose levels leads to the progressive impairment of insulin secretory response¹⁶⁹ and eventually culminates in apoptotic demise of beta cells.^{170,171,172,173,174} This paradigm of beta cell damage, also known as glucolipotoxicity, is believed to be associated with altered metabolism or “partitioning” of lipids to long-chain fatty acyl-CoA (LC-CoA) esters in lieu of their detoxification via mitochondrial oxidation.¹⁷⁵ LC-CoAs are the activated form of fatty acids that normally serve as substrates for carnitine palmitoyl transferase-1 (CPT-1) and undergo beta oxidation in mitochondria. However, under hyperglycemic conditions, these activated fatty acid esters accumulate in the cytoplasm and exert cytotoxic effects. The following sections highlight the molecular mechanisms underlying the shift in the metabolic fate of fatty acids and associated beta cell damage in T2D.

Hyperglycemia is marked by exaggerated glucose flux through mitochondria and diversion of glucose-derived carbons from the TCA cycle (cataplerosis) to the cytoplasm in the form of intermediates such as citrate.^{176,177} Citrate accumulation leads to inhibition of beta oxidation by giving rise to malonyl CoA, an inhibitor of

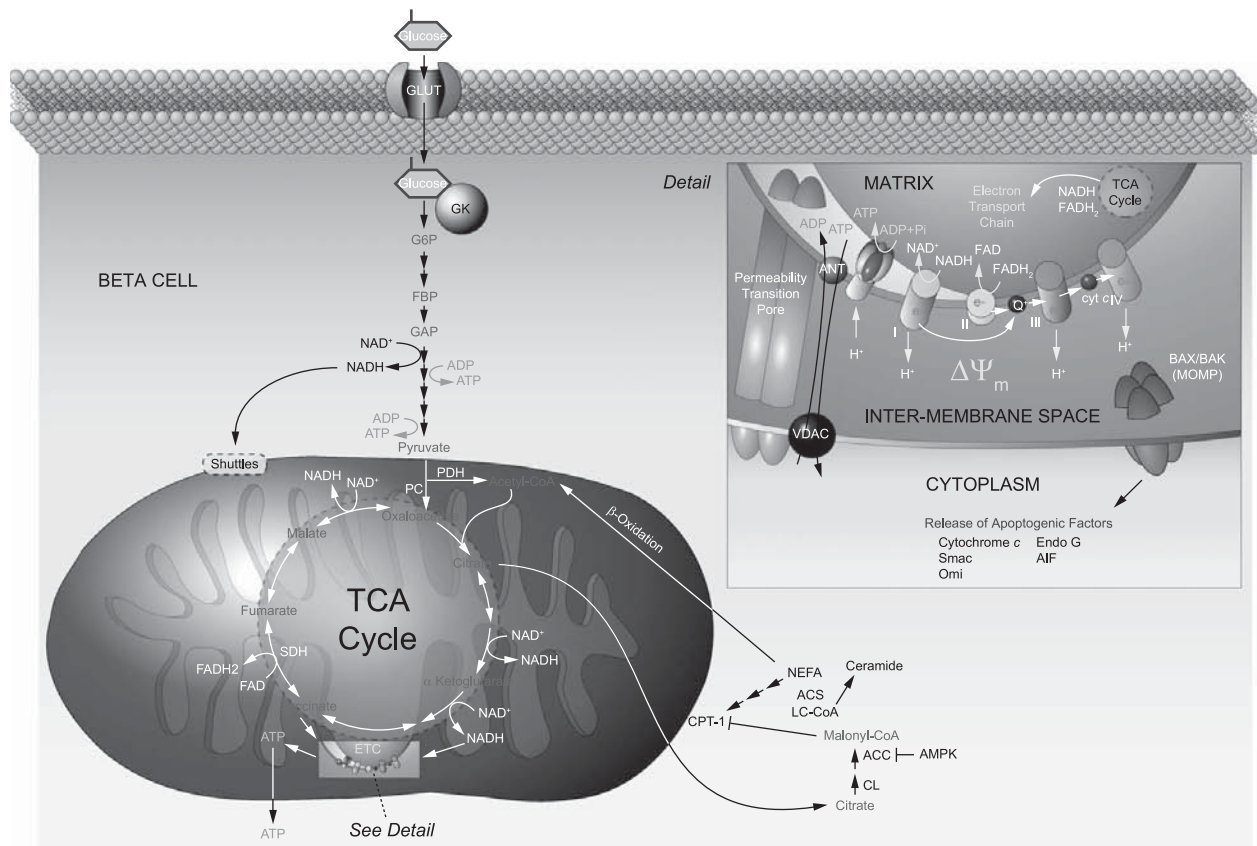


Figure 19-2. Shift in lipid partitioning associated with apoptosis in diabetic beta cells. Chronic exposure to glucose increases the cataplerotic flux of glucose-derived carbons from the TCA cycle and subsequent accumulation of citrate in the cytosol. Citrate serves as a precursor of malonyl CoA, an inhibitor of CPT-1 and beta oxidation. In addition to hyperglycemia, chronic exposure to fatty acids in the diabetic milieu further leads to accumulation of fatty acids in the cytosol, which in lieu of detoxification through mitochondrial beta oxidation, are esterified to form long-chain fatty acyl-CoA and exert cytotoxic effects on beta cells. ACC, acetyl-CoA carboxylase; ACS, acyl-CoA synthetase; CL, citrate lyase; CPT-1, carnitine palmitoyl transferase-1; ETC, electron transport chain; FBP, fructose 1,6-bisphosphate; GAP, glyceraldehyde phosphate; GK, glucokinase; GLUT, glucose transporter; G6P, glucose 6-phosphate; LC-CoA, long-chain acyl-CoA esters; NEFA, non-esterified fatty acids; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; SDH, succinate dehydrogenase. Image courtesy of Eric Smith of Dana-Farber Cancer Institute. See Color Plate 22.

CPT-1^{178,179} (Figure 19-2). In addition to malonyl CoA production, chronic elevation of glucose metabolism in this setting negatively regulates fatty acid oxidation by decreasing the cellular adenosine monophosphate (AMP)/ATP ratio and dampening AMP-activated protein kinase (AMPK), a cellular energy sensor that activates key enzymes necessary for mitochondrial metabolism of fatty acids.¹⁸⁰ Inhibition of fatty acid oxidation in turn leads to their accumulation in the cytosol and redirects their metabolism to esterification and ceramide production.^{41,174} The importance of this shift in lipid partitioning or channeling in lipotoxicity-induced death is corroborated by the protective effect of pharmacologic or genetic maneuvers that activate AMPK and CPT-1 or inhibit LC-CoA synthesis.^{170,181,182,183}

Several mechanisms have been implicated in death induced by glucolipotoxicity, including the effect of

fatty acids on the anionic phospholipid cardiolipin (CL), increased ceramide synthesis, and ROS production. Interestingly, these cytotoxic effects are only associated with saturated fatty acids.^{170,172} Monounsaturated fatty acids are metabolized to triglycerides and do not produce ceramide.^{171,184,185} Because cardiolipin is necessary for the attachment of cytochrome c to the inner mitochondrial membrane¹⁸⁶ and the assembly of higher order complexes of respiratory chains,¹⁸⁷ elevated fatty acids interfere with mitochondrial function and further facilitate cytochrome c egress from mitochondria in response to apoptotic stimuli.^{188,189} Consequently, impairment of CL synthesis in the presence of elevated fatty acids sensitizes beta cells to apoptosis.^{185,190,191}

Long-chain fatty acyl CoAs also serve as precursors for de novo synthesis of the lipid messenger ceramide.^{171,192} Apoptosis associated with glucolipotoxicity can be

blocked by inhibition of ceramide synthesis or overexpression of BCL-2.^{41,174,181} Notably, direct association of CPT-1 and BCL-2 was recently reported.¹⁹³ Whether the protective role of BCL-2 in glucolipotoxicity may be linked to its potential capacity to regulate fatty acid metabolism through CPT-1 is an intriguing thesis that remains to be experimentally tested. The proapoptotic effects of ceramide have been attributed to multiple downstream targets, including BAX conformational change,^{194,195} BID cleavage,¹⁹⁶ downregulation of PI3 kinase activity,^{197,198} BAD dephosphorylation,^{198,199} and transcriptional induction of BNIP3.²⁰⁰ However, the requirement of these proapoptotic BCL-2 proteins in glucolipotoxicity-induced apoptosis awaits loss-of-function studies. Interestingly, a recent report indicated that necrosis is an alternate form of death under glucolipotoxic conditions if execution of apoptosis is blocked by caspase inhibition.¹⁷⁰

The proapoptotic consequence of malonyl CoA and LC-CoA elevation in the context of glucolipotoxicity is intriguing because these same signals are transiently elevated under normal physiologic conditions of stimulatory nutrient/fuel concentration, serving as metabolic coupling factors and amplifying signals, respectively, to couple glucose metabolism and insulin exocytosis.^{201,202} Thus nutrient overload as in glucolipotoxicity appears to render a metabolic signaling pathway, which is normally operative during physiologic control of insulin secretion, into a proapoptotic signal.

3.3.2. Endoplasmic reticulum stress

The ER in beta cells has evolved to efficiently handle synthesis, folding, processing, and export of large amounts of newly synthesized insulin, endowing beta cells with a high secretory capacity. Unfolded protein response (UPR) is an adaptive quality-control mechanism executed by the ER that ensures proper refolding of misfolded proteins or degradation of those that are not correctly folded or processed. Beta cells are especially sensitive to UPR because they heavily rely on ER and Ca^{2+} for proper protein folding and secretion of insulin granules.^{203,204} In beta cells, UPR can be induced by toxic oligomers of islet amyloid polypeptide (IAPP; also known as amylin), glucolipotoxicity, oxidative stress, cytokines, hypoxia, and reduced protein glycosylation.²⁰⁵ Prolonged UPR transforms into a proapoptotic stress response (ER stress) when the homeostatic folding of newly synthesized proteins is not achieved. ER stress can also be associated with genetic/environmental factors and aberrations in Ca^{2+} homeostasis that compromise the proper function of ER.²⁰⁵ Insulin resistance can

lead to ER stress in the beta cell as a state in which the demand for insulin secretion and the risk of ER overload simultaneously increase. Furthermore, because beta cell mass is progressively lost in both type 1 and type 2 diabetes, the remaining beta cells become prone to ER stress because of ever-increasing insulin demand. The following sections focus on the inducers of ER stress in diabetic beta cells, especially the pathophysiology of IAPP and the underlying apoptotic mechanisms.

IAPP is a 37-amino acid peptide processed and cosecreted with insulin²⁰⁶ that may carry physiologic roles in control of food intake²⁰⁷ and paracrine inhibition of insulin secretion by beta cells.^{208,209} IAPP has a high capacity to form insoluble toxic oligomers²¹⁰ and constitutes a major inducer of ER stress in beta cells. During progression of T2D, with the increase in insulin demand, beta cells synthesize more IAPP to cosecrete with insulin. However, the increase in IAPP expression is much higher than insulin in this case,²¹¹ and the capacity of the ER to properly execute protein folding is eventually saturated. Consequently, toxic oligomers of IAPP accumulate leading to beta cell degeneration.^{212,213,214} Remarkably, IAPP oligomers exhibit similar three-dimensional structure to that of amyloid A β peptide in Alzheimer's disease, α -synuclein in Parkinson's disease, polyglutamine in Huntington's disease, and prions, despite amino acid sequence differences.^{215,216} Indeed, an antibody raised against A β oligomers recognizes IAPP oligomers.²¹⁵ Thus IAPP-associated beta cell loss in T2D may share common pathophysiology with neuronal loss induced by amyloidogenic proteins in neurodegenerative disorders; notably, the contribution of ER stress-induced apoptosis to disease progression.^{53,217,218} Transgenic expression of human IAPP (hIAPP) in mice or rats is associated with elevated beta cell apoptosis, decreased beta cell mass, and hyperglycemia in a gene dosage-dependent manner.^{212,219,220} Furthermore, IAPP oligomers^{221,222} and ER stress markers can be selectively detected in pancreatic biopsies from T2D individuals compared with control samples.^{223,224,225}

The sensors and effector pathways in charge of executing UPR and ER-stress associated apoptosis have been recently reviewed.^{205,226} Briefly, three protein sensors, PERK (protein kinase-like ER kinase), ATF6 (activating transcription factor 6), and IRE1 (inositol requiring 1) are triggered in response to unfolded proteins and activate an adaptive program that reduces production of new client proteins for the ER folding machinery, helps refold misfolded proteins, and degrades protein aggregates. UPR initiates with PERK, which, on activation, phosphorylates the translation initiation factor eIF2 α , leading to inhibition of general protein

translation and selective increase in ATF-4 translation (Figure 19-3a). The transcription factor ATF-4 in turn increases expression of select chaperones and antioxidant defense genes. Another UPR sensor, IRE1, is activated by dimerization and transphosphorylation, leading to stimulation of its inherent endoribonuclease activity and processing of mRNA encoding the transcription factor XBP-1 (X-box binding protein-1). XBP-1, together with ATF-6, regulates transcription of additional genes required for UPR, including chaperones, ER-associated degradation (ERAD) components, and autophagy genes (Figure 19-3a). Increased ERAD components and autophagy help clear unfolded protein, protein aggregates, and damaged organelles.²²⁷ Accumulation of unfolded proteins also leads to the release of ER Ca^{2+} , which activates a signal transduction program mediated by Ca^{2+} /calmodulin-dependent kinase β , leading to enhanced autophagy^{228,229} (Figure 19-3a). If the integrated outcome of these signaling pathways does not resolve the ER load of unfolded and aggregated proteins, then these same sensors can engage the intrinsic pathway of apoptosis.²³⁰ Consistent with the notion that UPR is primarily an adaptive mechanism evolved to preserve cellular survival, ablation of *Perk* in beta cells is associated with significantly higher sensitivity to apoptosis on stress associated with unfolded proteins and nutrient overload.^{231,232,233} Consistent with these findings, interference with eIF1 α phosphorylation downstream of PERK is also associated with loss of beta cell mass.²³⁴ Importantly, polymorphisms in several genes associated with UPR and ER stress, such as PERK,^{235,236,237,238} ATF 6,^{239,240} and IAPP,²⁴¹ are associated with diabetes in humans.

Apoptotic pathways downstream of ER stress are under active investigation and involve both transcriptional and post-translational mechanisms (Figure 19-3b). p53 and C/EBP homologous protein (CHOP)/growth arrest and DNA damage induced gene-153 (GADD153), a transcription factor induced by ATF4, initiate an ER stress-associated transcription program that is marked by changes in expression levels of several BCL-2 family members, including downregulation of BCL-2²⁴² and upregulation of BIM,²⁴³ NOXA, and p53-upregulated modulator of apoptosis (PUMA).^{244,245} Furthermore, CHOP has been implicated in increased expression of death receptors such as FAS and DR5^{246,247} and attenuation of AKT survival pathway through augmented expression of its inhibitor TRB3.²⁴⁸ Loss of CHOP protects beta cells from ER stress induced by NO or IAPP and associated diabetes.^{223,249,250} Downstream of IRE1, TRAF-2 modulates the apoptotic response to ER stress by multiple mechanisms, such as

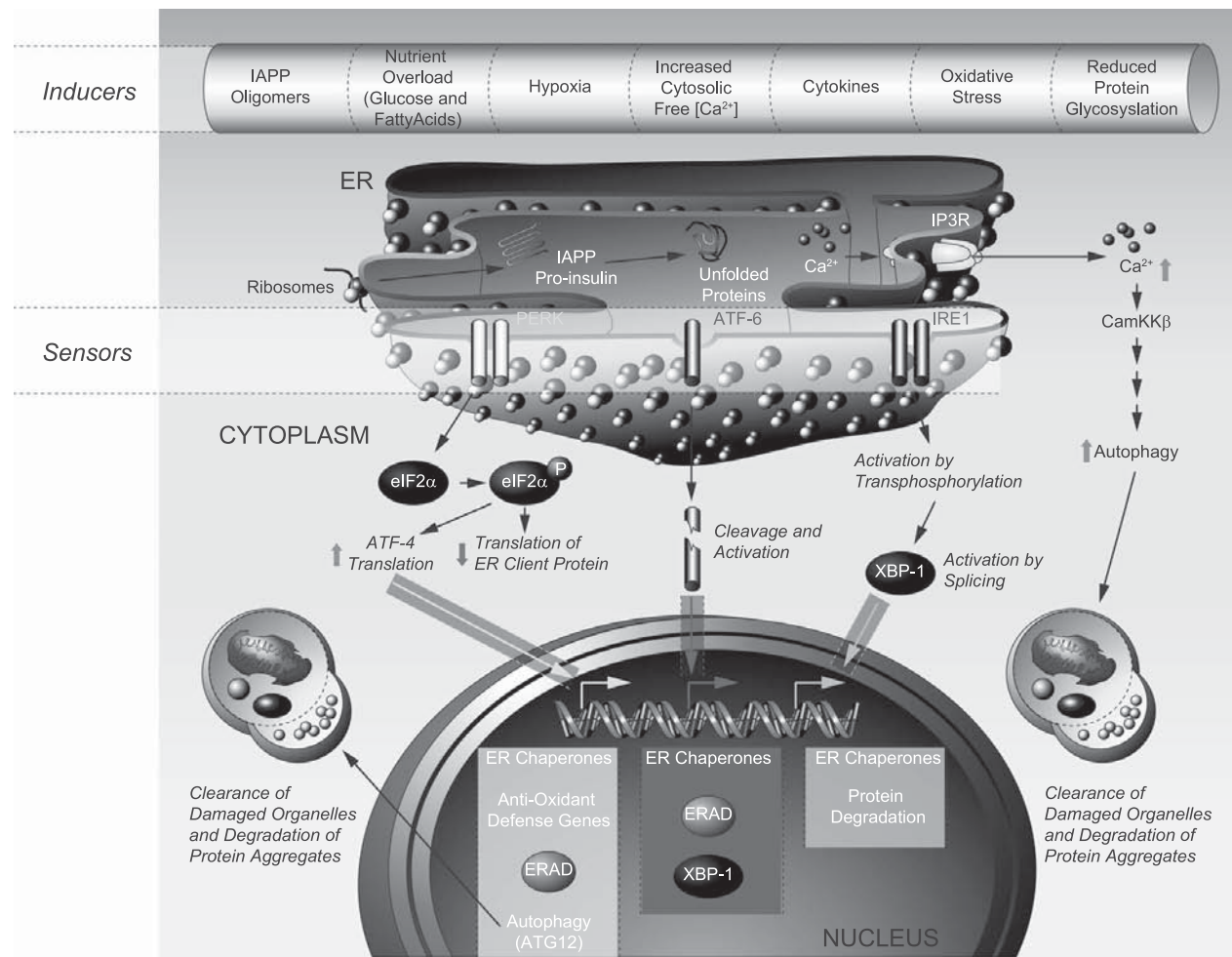
activation of ER-linked caspases²⁵¹ (Figure 19-3b). Alternatively, TRAF-2 is recruited to IRE1²⁵² and mediates c-Jun N-terminal kinase (JNK) activation through apoptosis signal-regulating kinase (ASK-1).²⁵³ JNK-1 phosphorylation of BCL-2 inhibits its survival function.²⁵⁴ Beyond the transcriptional and post-translational mechanisms that impinge on BCL-2 family members on ER stress-induced apoptosis, select members of this family can functionally interact with the ER by regulating Ca^{2+} homeostasis²⁵⁵ and IRE1 activation.²⁵⁶

4. BETA CELL APOPTOSIS AND ISLET TRANSPLANTATION THERAPY

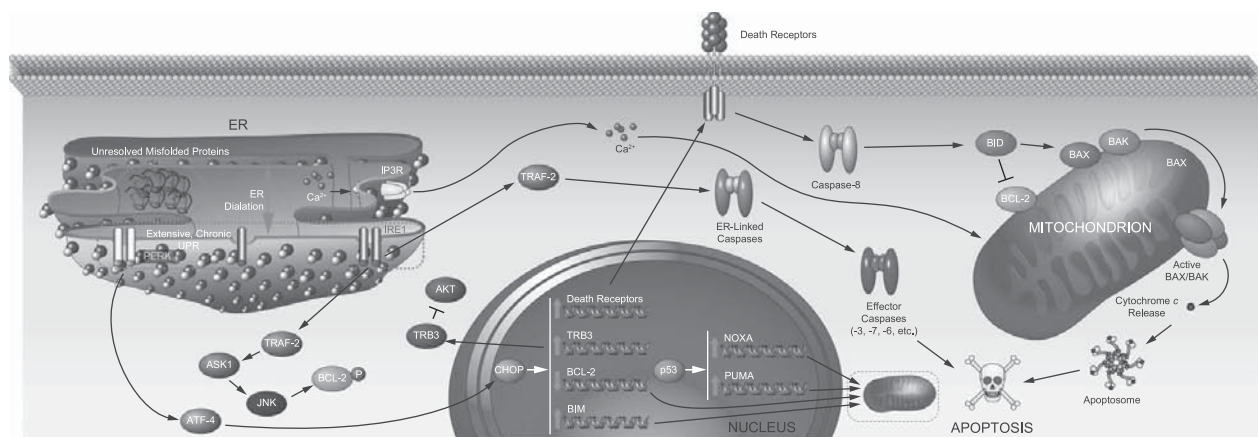
Because loss of functional beta cell mass is central to the etiology of diabetes, beta cell transplantation is being actively pursued as a possible therapeutic approach.^{257,258} The success of transplantation therapy, however, has been limited because of an insufficient source of insulin-producing tissue available for transplantation and the loss of islet viability during isolation or expansion and immediately after transplantation. These therapeutic challenges have spurred active search for strategies to enhance beta cell viability and improve “engraftment” of transplanted islets.

Islet viability during isolation or expansion and shortly after transplantation is compromised by hypoxia as a result of loss of the normal vascularized islet microenvironment. On revascularization, islets undergo further oxidative stress.^{259,260} In addition to this metabolic stress, islets are subject to immune-mediated damage. In response to tissue trauma during surgery and ischemic reperfusion, donor islets produce chemokines that activate an innate immune response in the host marked by release of inflammatory cytokines such as TNF- α , IL-1 β , and IFN- γ ,²⁶¹ compromising the viability of donor islets.²⁶² Furthermore, only a small percentage of transplanted islets that survive under these conditions display physiologic insulin secretory characteristics.^{263,264,265}

Multiple strategies have been explored to attenuate apoptosis during islet transplantation.²⁶⁶ Expression of antioxidant enzymes such as glutathione peroxidase and superoxide dismutase alleviate oxidative damage associated with hypoxia and reoxygenation.²⁶⁷ Inhibition of signaling downstream of inflammatory cytokines by IL-1 receptor antagonists^{268,269,270} or inhibition of the JAK-STAT pathway through SOCS proteins^{78,131,271} protects islet grafts. Studies in preclinical models of islet transplantation using both rodent and human islets have also shown that combined inhibition of the extrinsic and intrinsic pathway by blocking effector caspases through



(a)



(b)

Figure 19-3. Signaling pathways in response to unfolded proteins. **(a)** The unfolded protein response (UPR) in beta cells is activated by stimuli such as islet amyloid polypeptide (IAPP, amylin), nutrient overload, and oxidative stress and triggers an adaptive response through sensors that include PERK, ATF-6, and IRE1. Through changes in protein translation and gene expression, UPR leads to refolding of misfolded proteins, degradation of protein aggregates, and restoration of ER protein folding homeostasis. **(b)** ER stress ensues on prolonged UPR and unresolved protein aggregates through the same UPR sensors. CHOP, an ATF-4 dependent gene downstream of PERK, compromises beta cell survival by altering the expression levels of several BCL-2 family members and inhibiting the PI3 kinase signaling pathway, whereas TRAF-2 mediates the apoptotic response downstream of IRE1 through activation of ER-linked caspases and JNK. Image courtesy of Eric Smith of Dana-Farber Cancer Institute. See Color Plate 23.

XIAP,^{272,273,274,275} survivin,²⁷⁶ or pan-caspase inhibitor z-VAD.fmk²⁷⁷ imparts potential therapeutic benefits. Other attempts at interfering with the mitochondrial pathway of apoptosis during islet transplantation include over-expression of BCL-2 or BCL-X_L in islets. Although BCL-2 or BCL-X_L protect islets against hypoxia and apoptosis induced by inflammatory cytokines^{278,279} and display potential benefit in islet transplantation,²⁸⁰ their efficacy in improving long-term engraftment and euglycemia may be more complex than anticipated. Specifically, although it prevents apoptosis, over-expression of BCL-X_L is associated with elevated rates of Ca²⁺ leak from the endoplasmic reticulum²⁸¹ and impairment of insulin secretion in beta cells.²⁸² Thus the advantages of antiapoptotic molecules in beta cells will have to be carefully assessed in light of their other homeostatic functions. A potential dual benefit in preservation of a functional beta cell mass in this setting may be inherent in another BCL-2 family protein, BAD. BAD phosphorylation on Ser155 within its BH3 sequence serves as a molecular switch that inactivates its proapoptotic function while enabling its capacity to activate glucokinase (GK) and stimulate insulin secretion.^{44,283} Both GK²⁸⁴ and BAD⁴⁴ regulate beta cell mass compensation. Importantly, BAD phosphorylation is sensitive to glucose²⁸³ and the nutritional (fed/fasted) state.⁴⁴ BAD phosphorylation is also modulated by GLP-1,²⁸⁵ an incretin hormone known to stimulate both beta cell survival and insulin secretion²⁸⁶ and shown to impart therapeutic advantage in islet transplantation.^{287,288,289} These observations suggest that BAD phosphorylation is integrated with nutrient and hormonal regulation of beta cell mass and function and warrant investigation whether genetic or pharmacological simulation of BAD function through BAD BH3 phosphomimetic compounds may improve islet transplantation.^{44,290} Although the aforementioned strategies in preserving islet grafts during transplantation may be promising, additional challenges in transplantation therapy include translation of preclinical models to clinic and achievement of long-term islet engraftment.

5. SUMMARY

The very cellular features that enable beta cells to be efficient secretory machines – including their critical reliance on mitochondrial metabolism, proper intracellular Ca²⁺ flux, and the capacity to synthesize, process, and secrete large amounts of insulin – render them especially susceptible to damage induced by mitochondrial malfunction, bioenergetic decline, oxidative stress, and nutrient overload. Apoptosis plays a major role in both

physiologic remodeling of endocrine pancreas and in progressive deficiencies of beta cell mass associated with diabetes. Because loss of beta cell viability presents a significant challenge in the quest for preservation of functional beta cell mass through beta cell regeneration and replacement therapy, strategies that sustain the survival of these cells will be instrumental in reversing diabetic outcomes.

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Apoptosis in the Physiology and Diseases of the Respiratory Tract

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The lung provides a huge contact interface between the organism and its environment. Its mucosal surfaces must permit gas exchange between the blood and air, but also act as a barrier against a plethora of microorganisms. In addition, inhaled toxins and particles may enter the organism via the lung. Accordingly, inflammatory airway and lung diseases are among the most prevalent human morbidities. Lung cancer, which in most cases can be attributed to tobacco smoking, is the leading cause of cancer-related mortality in the developed world. In this chapter, we summarize the role of apoptotic cell death in lung development and in clinical disease states.

1. APOPTOSIS IN LUNG DEVELOPMENT

The physiology and genetics of the branching program underlying lung development are just being unraveled. Following the initial separation of the lung bud from the prospective esophagus in the embryonic stage, apoptosis of mesenchymal and epithelial cells can be observed during the various stages of lung development. Regulation of developmental apoptosis has been linked to the expression of cytokines, such as transforming growth factor- β 1 (TGF- β 1) and insulin-like growth factor 1 (IGF-1), as well as additional apoptosis-related proteins and nitric oxide. In the early stages of lung development, apoptosis is mainly detectable in the mesenchymal tissue layer. Indeed, apoptosis was almost exclusively found in the regions of new bud formation or in the mesenchyme underlying branch points, thus providing space for the outgrowth of lung buds. During later stages of lung development, both alveolar epithelial and mesenchymal tissue apoptosis can be detected. At this time, apoptosis coincides with airway branching, decreased cell proliferation, and alveolar epithelial thin-

ning, thus implying cellular apoptosis as a significant contributor of lung remodeling. Alveolarization and microvascular maturation do not stop at birth, but continue up to a few years after birth(1). After birth, apoptosis emerges as an important process after extensive proliferation of type 2 alveolar epithelial cells, which are produced in higher numbers than actually required. Consequently, type 2 cells are removed either by differentiation or by apoptosis, thus preserving functional alveoli.

Using different approaches, genes encoding most of the core factors of the apoptotic machinery have been targeted in the mouse. Although some of these gene-targeted mice exhibited developmental defects, none of them showed particular pathology in the respiratory tract or lung. This observation does not rule out an involvement of apoptosis regulators in lung physiology, as some of these knockout mice, such as those deficient in Mcl-1, cytochrome c, caspase-8, casper, or FADD, succumb during embryonic development, and their lung development cannot be examined. Recently, mice with targeted deletion of the miR-17~92 microRNA were described to exhibit embryonic lethality due to lung hypoplasia and cardiac defects. Examination of lung tissues revealed increased expression of the proapoptotic BH3-only protein Bim, and miR-17~92 was found to transcriptionally repress Bim. Overall, these findings suggest that apoptosis plays an important role in mammalian lung development.

2. APOPTOSIS IN LUNG PATHOPHYSIOLOGY

2.1. Apoptosis in pulmonary inflammation

The lung is characterized by a large mucosal surface at risk for exposure to many types of microorganisms (e.g., viral, bacterial) and irritants (e.g., ozone), which

can lead sometimes to severe inflammatory reactions. Apoptotic cells are usually rapidly and effectively cleared from the lung so that no apoptotic cells are detectable in the healthy lung. Even during extensive inflammatory reactions, (e.g., during lung infection), few apoptotic cells are found in lung tissue. Neutrophils derived from broncho-alveolar lavage (BAL) samples of patients with pneumonia even display decreased rates of apoptosis. Also, during resolution of lung inflammation, apoptotic cells are rapidly removed by macrophages through cell recognition receptors (phosphatidylserine receptor, CD36, and α v integrins). Uptake of apoptotic cells by macrophages then leads to a noninflammatory environment by production of anti-inflammatory mediators that include TGF- β , interleukin-10 (IL-10), and prostaglandin E2 (PGE2). Indeed, in animal studies, instillation of apoptotic cells into the lung results in rapid resolution of inflammatory responses, suggesting that apoptosis and uptake of apoptotic cells provides an intrinsic anti-inflammatory circuit that attenuates proinflammatory responses. However, in several lung diseases, increased numbers of apoptotic cells have been described in the airways and lung tissue, implying either an increase in apoptosis or defects in clearance of apoptotic cells in pathophysiology. In cystic fibrosis, which is characterized by a massive influx of inflammatory cells and release of proteases in the lung, increased numbers of apoptotic neutrophils are detectable in the airway. This finding has been linked to cleavage of the phosphatidylserine receptor on macrophages by neutrophil elastase, which impairs the uptake of apoptotic cells and contributes to ongoing inflammation. Similarly, alveolar macrophages from patients with chronic obstructive pulmonary disease (COPD) are less effective in phagocytosing apoptotic airway epithelial cells as compared with controls. Also, increased apoptosis of lung structural cells has been described in several lung diseases, including acute lung injury, COPD, and lung fibrosis.

2.2. Apoptosis in acute lung injury

Acute lung injury (ALI) and the more severe acute respiratory distress syndrome (ARDS) represent clinical syndromes that result from complex responses of the lung to a multitude of direct and indirect stresses. Important pathophysiologic changes found in patients with ALI are alveolar inflammation and injury. Indeed, epithelial injury is one of the hallmarks of ALI in patients, and alveolar epithelial cells are especially affected. Similar to patients with pneumonia, only few apoptotic inflammatory cells are found in the lung, probably due to increases

in growth factors and rapid uptake of apoptotic granulocytes by macrophages. In contrast, it has recently become clear that increased apoptosis is detectable in parenchymal lung cells of adult patients with ALI and ARDS. Similar observations have also been reported in newborns with acute lung injury. Especially alveolar epithelial cells have been found to be apoptotic, leading to increased epithelial permeability and subsequent alveolar flooding. The increase in alveolar cell apoptosis could be mediated by increased levels of soluble Fas ligand (sFasL), which can be detected in BAL samples of patients with ALI and ARDS. During onset of ARDS, sFasL is highly biologically active and induces apoptosis of alveolar epithelial cells *in vitro*, particularly affecting distal epithelial cells. Also, in patients with ARDS, Fas expression on alveolar epithelial cells that line the alveolar walls is increased. Together, these findings are strongly suggestive that activation of the Fas pathway is an important contributor to alveolar epithelial cell apoptosis leading to the development of ALI and ARDS, in addition to other factors such as mechanical stress, hyperoxia, and hypoxia.

Animal models of ALI have confirmed that apoptosis of parenchymal lung cells contributes to acute lung damage. Indeed, direct instillation of sFasL into the lung or administration of an activating anti-Fas antibody leads to increased alveolar cell apoptosis associated with increased pulmonary inflammation. Also, meconium instillation into the lung, as a model for acute lung injury in newborns, leads to increased apoptosis of epithelial cells. Additionally, models of lung hyperoxia have revealed that apoptosis is a prominent component of the acute response, which is also mediated by Fas/FasL pathway. Other models of ALI involve instillation of bacterial lipopolysaccharides (LPS) into the lung. In these models, increased apoptosis of alveolar epithelial cells and interstitial inflammatory cells are also detectable. In addition to epithelial cell apoptosis, endothelial cell apoptosis can be found in models of hemorrhagic shock. Interestingly, administration of blocking anti-Fas antibodies or caspase-3 inhibitors attenuates lung injury after LPS instillation, suggesting that regulation of apoptosis could be a potential treatment of ARDS.

However, apoptosis also has beneficial effects during the resolution of inflammation after acute lung injury. Indeed, during resolution of ALI, hyperplasia of type II pneumocytes occurs as a reparative phenomenon. During clearance of inflammation, extensive apoptosis of type II pneumocytes mediated by Fas/FasL accounts for the disappearance of these cells from the lung. Therefore, although blocking apoptosis at an early stage of the disease might be beneficial for the course of ALI

and ARDS, inhibiting apoptosis at later stages might be counter-indicated because apoptosis and engulfment of apoptotic cells are important events during resolution of inflammation.

2.3. Apoptosis in chronic obstructive pulmonary disease

COPD is a chronic respiratory disease characterized by airflow limitation that is not fully reversible. The airflow limitation usually is progressive and associated with inflammation of the lungs. COPD is a combination of two phenotypes, chronic obstructive bronchitis, defined clinically as chronic productive cough in combination with airflow obstruction, and emphysema, characterized pathologically as the presence of permanent enlargement of the airspaces distal to the terminal bronchioles, accompanied by destruction of their walls. COPD is the result of exposure to noxious particles. Cigarette smoke is the major risk factor for the development of this disease in the Western world. However, despite progress in the characterization of COPD, so far the pathophysiologic cause has not been identified. Several mechanisms have been suggested to contribute to the development of COPD. Airway inflammation, oxidative stress, a disrupted balance between proteolytic and antiproteolytic molecules in the lung, as well as premature aging and senescence are suspected to contribute to the development of COPD. In recent years, alternative mechanisms have been discussed. There is increasing evidence from studies in humans as well as data from animal models that defective homeostasis of apoptosis and proliferation in the lung might lead to a disruption of alveolar architecture, leading to emphysema.

Indeed, several studies in lung tissue samples from patients with COPD have described increased apoptosis as compared with controls. Increased apoptosis was found in several types of lung cells, including endothelial cells, interstitial cells, and alveolar epithelial cells. In addition, increased detection of active caspase-3 and expression of Bax and Bad as well as elevated levels of airway granzyme B and perforin have been reported, providing evidence for enhanced activation of proapoptotic pathways in these patients.

Animal models of COPD have also suggested an important role of apoptosis in the development of emphysema, even in the absence of significant inflammatory changes. Experimental observations, which describe alveolar enlargement as a result of alveolar and endothelial cell apoptosis, have supported the concept of a direct involvement of apoptosis in emphysema development. Direct targeting of alveolar cells in mice by intratracheal

administration of active caspase-3 or ceramide results in epithelial apoptosis and development of emphysematous changes in mice. Inhibition of growth factor signaling in the lung also results in increased apoptosis. Indeed, targeting vascular endothelial growth factor (VEGF) or VEGF receptor (VEGFR) in the lung increases apoptosis of alveolar cells, leading to enlargement of the alveolar space. This process can be attenuated by treatment with a caspase inhibitor, thus preventing apoptosis and development of emphysema. Similar to animal studies, decreased expression of VEGF and VEGFR has been detected in patients with emphysema, suggesting that epithelial and endothelial alveolar septal death due to a decrease of endothelial cell maintenance factors contributes to the pathogenesis of emphysema. Some studies have linked increased apoptosis with other pathophysiologic mechanisms of emphysema development (such as protease/anti-protease imbalance). Mice that over-express interferon γ in the lungs develop emphysema and have increased numbers of apoptotic parenchymal lung cells. Interestingly, inhibition of cathepsin S, an important elastase in the lung, results in decreased apoptosis and emphysema in interferon-expressing transgenic mice. This suggests that protease-dependent epithelial cell apoptosis is a critical event in the pathogenesis of alveolar remodeling and emphysema, linking apoptosis to protease/anti-protease imbalance. Overall, these findings show that apoptosis of lung cells plays an important role during the development of COPD and especially emphysema. Thus far, no therapeutic approaches have been clinically developed to prevent apoptosis in this patient group.

2.4. Apoptosis in interstitial lung diseases

Interstitial lung diseases constitute a heterogeneous collection of diseases that are characterized by a progressive distortion of the alveolar architecture and replacement by fibrotic tissue. The clinically most relevant form is idiopathic pulmonary fibrosis (IPF), which is characterized by progressive dyspnea, decline in lung function, and death within 3 to 4 years from diagnosis. Histopathologically, IPF typically shows a pattern of usual interstitial pneumonia, the cardinal features of which are patchy fibrosis with variable numbers of fibroblastic foci interspersed with areas of normal or nearly normal lung. Although initial studies focused on the role of inflammation in inciting fibroblast activation and fibrosis, current concepts suggest a central role of the epithelium in IPF pathogenesis. It has been proposed that IPF is the result of ongoing alveolar epithelial

injury and subsequent dysregulated repair associated with the formation of fibroblast-myofibroblast foci, which evolve into fibrosis. A relatively small increase in the rate of epithelial cell apoptosis is predicted to result in considerable cell loss over time and thus minor upregulation of epithelial apoptosis, especially of alveolar epithelial cells, could account for excessive epithelial loss. Therefore, it is likely that apoptosis of epithelial cells is important in the injury and repair processes present in IPF. Indeed, alveolar epithelial cell injury and apoptosis were found in the lungs of patients with IPF, even in areas that histologically seemed normal. Especially in areas where epithelial cells are in close proximity to myofibroblasts, increased epithelial cell apoptosis is frequently detectable. In addition, lung tissues from IPF patients exhibit increased expression of proapoptotic proteins (p53, Bax, and caspase-3) and decreased expression of antiapoptotic proteins (Bcl-2) in epithelial cells.

Animal studies have also demonstrated that apoptosis of alveolar epithelial cells is sufficient to induce pulmonary fibrosis. Intratracheal instillation of activating anti-Fas antibody induces apoptosis of alveolar epithelial cells and results in pulmonary fibrosis in rodents. Apoptosis is also induced by over-expression of TGF- β in the rodent lung, leading to inflammation, myofibroblast hyperplasia, tissue fibrosis, and honeycombing. Treatment with caspase inhibitors markedly ameliorates fibrosis and alveolar remodelling. In addition, apoptosis of alveolar epithelial cells is detected. In the commonly used bleomycin-induced pulmonary fibrosis model, direct inhibition of apoptosis by blocking the Fas/FasL pathway results in a blunted apoptotic response to bleomycin and decreased collagen production. Also, treatment with caspase inhibitors not only prevents apoptosis, but also reduces the histopathological grade of lung inflammation and decreases fibrosis.

2.5. Apoptosis in pulmonary arterial hypertension

Pulmonary hypertension (PH) is a hemodynamic and pathophysiological condition defined as an increase in pulmonary artery pressure ≥ 25 mmHg at rest as assessed by right heart catheterization. Pulmonary arterial hypertension (PAH) is a clinical condition characterized by the presence of pre-capillary PH in the absence of other causes of pre-capillary PH such as PH due to lung diseases, chronic thromboembolic PH, or other rare diseases. PAH includes different forms that share a similar clinical picture and virtually identical pathological changes like vasoconstriction, in situ thrombosis, and vascular remodeling of pulmonary arteries (Gaile et al. 2009).

PAH can be classified into five categories: (1) idiopathic PAH, (2) heritable PAH, (3) drug- or toxin-induced PAH, (4) PAH associated with other diseases (e.g. connective tissue diseases, HIV infection, portal hypertension), and (5) persistent pulmonary hypertension of the newborn. PAH is a progressive disease characterized by abnormal muscularization of distal pulmonary arteries, striking reduction in arterial numbers, progressive intimal hyperplasia leading to occlusive changes in the pulmonary arteries, and so-called plexiform lesions. The initial pathological events are thought to be related to dysregulation of pulmonary artery smooth muscle proliferation. Increased proliferation and decreased apoptosis of pulmonary arterial smooth muscle could mediate thickening of the pulmonary vasculature, which subsequently would lead to reduced inner diameter and increased pulmonary vascular resistance. Several lines of evidence have suggested an impaired regulation of pulmonary artery smooth muscle proliferation. In a subset of PAH patients, loss-of-function mutations in bone morphogenetic protein receptor 2 (BMPR2) has been found. Activation of the BMPR2 pathway results normally in suppression of arterial smooth muscle cell proliferation. In contrast, arterial smooth muscle cells from patients with PAH were not inhibited in their proliferation after BMPR2 activation. Also, mediators that favor suppression of apoptosis (e.g., Bcl-2) are upregulated in lung vessels of patients with PAH. These findings suggest that some abnormalities described in PAH contribute to resistance to apoptosis and a proliferation/apoptosis imbalance within the vascular wall, thus leading to smooth muscle proliferation and vascular remodeling. These hypotheses are supported by the description of increased expression of the Survivin protein in remodeled pulmonary arteries from patients with PAH. In this regard, dysregulated Survivin expression is considered to be a major pathological mechanism in animal models of PAH, which have demonstrated Survivin over-expression coinciding with pulmonary vascular remodeling. Furthermore, inhibition of Survivin by gene therapy in these models resulted in pulmonary artery smooth muscle cell apoptosis and decreased pulmonary vascular resistance, heart failure, and vascular remodeling. These findings suggest that targeted pro-apoptotic agents could be a possible new therapeutic approach for patients with PAH.

2.6. Apoptosis in lung cancer

Lung cancer is the leading cause of cancer mortality in the United States and Western Europe. The main risk factor for the development of lung cancer is inhaled tobacco exposure. Smoking 20 cigarettes per day for

20 years (i.e., 20 pack-years) increases the age-adjusted risk for lung cancer approximately 20-fold. In the light of the high prevalence of tobacco smoking in Asia, Eastern Europe, Latin America, and South America, lung cancer seems destined to become an even larger global health problem, with enormous socioeconomic and health care costs. Based on histology and clinical course, lung cancers have been grouped into small-cell lung cancer (SCLC), which comprises less than 20% of lung cancer cases, virtually all of which are associated with cigarette smoking, and non-small-cell lung cancers (NSCLC), which constitute more than 80% of lung cancer cases. The latter is a heterogeneous group composed of several histologies, such as squamous cell carcinoma (SCC), adenocarcinoma, large-cell carcinoma, bronchoalveolar carcinoma, and others. Historically, all NSCLCs have been uniformly treated. However, more recently it has been recognized that some NSCLC subgroups are more susceptible to certain therapies. For example, patients with adenocarcinoma and no smoking history have a high incidence of amplification and mutations of the epidermal growth factor receptor (EGFR), which make them prone to responding to EGFR tyrosine kinase inhibitors (TKIs), such as erlotinib or gefitinib. Also, the antifolate pemetrexed achieved superior survival outcomes in patients with adenocarcinoma and large-cell carcinoma, but not those with SCC. Further, SCC patients are excluded from receiving the anti-VEGF antibody bevacizumab in combination with chemotherapy because of a higher risk of bleeding complications. These clinical and histological distinctions are currently substantiated by more sophisticated efforts of tumor characterization, such as gene expression profiling and massively parallel sequencing. Hence it is expected that molecular predictors and specific targets will play an even larger role in future treatment decisions for patients with lung cancer.

Against this background, the analysis of apoptosis pathways in lung cancer is of particular importance. First, as in most cancers, deregulation of apoptosis is an important event in lung carcinogenesis. This is exemplified by inactivating mutations of the *TP53* tumor suppressor gene, which are found in approximately half of lung cancers. Accordingly, loss of p53 accelerates tumor development in a mouse model of K-ras-induced lung carcinogenesis. p53 gene transfer and pharmacological restoration of p53 function have been shown to induce apoptosis in p53-defective lung cancer cells in vitro and in pilot studies of somatic gene therapy in vivo. Additional genes involved in apoptosis regulation were found to be differentially expressed in murine lung cancer models as well as in human lung cancer samples as compared with nonmalignant tissues. In addition,

hypothesis-driven studies have specifically addressed apoptosis regulators with known function. For example, in a transgenic model of Raf-induced lung carcinogenesis, the onset of tumor development was greatly delayed by targeted deletion of Bcl-2. Accordingly, protein expression patterns of various pro- and antiapoptotic Bcl-2 family proteins correlated with prognosis in surgically resected lung cancer patients in some studies, in addition to changes in caspase expression. Taken together, these studies support a role of apoptosis in the development and progression of lung cancer. Hence apoptosis-directed strategies merit examination for lung cancer prevention and treatment. Indeed, at present, a chemical inhibitor of antiapoptotic Bcl-2 family proteins (BH3 mimetic) is currently in clinical testing for SCLC. Prospective studies are needed to define a role for expression analysis of apoptosis regulators as prognostic factors or predictors for treatment decisions in lung cancer patients.

Second, cytotoxic chemotherapy and gamma radiation, which are both thought to exert at least some of their activities by inducing apoptosis, still provide the basis of current standard treatment protocols for patients with nonresectable advanced and metastatic lung cancers. In addition, EGFR mutations in lung cancer were shown to activate antiapoptotic pathways, and this was reversed by EGFR TKI treatment. Hence deregulation in apoptotic signal transduction could interfere with the therapeutic activity of lung cancer therapies. Alternatively, proapoptotic therapies could overcome resistance to current drugs in clinical use for NSCLC and SCLC patients. Expression of antiapoptotic Bcl-2 family proteins was found to interfere with drug sensitivity of cancer cells, which is overcome by gene transfer-mediated expression of proapoptotic Bcl-2 proteins. These studies provide a lead for the development of pharmacological compounds targeting anti-apoptotic Bcl-2 proteins in lung cancer. Of particular interest are so-called BH3 mimetics, which exhibit promising activity in preclinical models of SCLC and NSCLC. Additional apoptotic targets have been identified that could enhance the efficacy of cytotoxic lung cancer therapies. The mitochondrial protein Smac is thought to interfere with the inhibition of caspases by inhibitor of apoptosis (IAP) proteins. Accordingly, Smac-derived peptides were shown to sensitize lung cancer cells to cytotoxic anticancer drugs in vitro. However, recent studies with small-molecule IAP inhibitors suggest a role in tumor necrosis factor (TNF) signaling, in addition to allowing caspase activation. Conditional expression of pp32/PHAPI, a putative modulator of apoptosome activity, sensitizes drug-resistant lung cancer cells to apoptosis in vitro and in vivo. Interestingly, high expression of pp32/PHAPI

in tumor biopsies correlates with improved outcome of NSCLC patients undergoing chemotherapy. Hence the functionality of apoptotic signal transduction pathways appears to determine – at least in part – the preclinical and clinical efficacy of cytotoxic and molecularly targeted lung cancer therapies.

Although most, if not all, current anticancer drugs primarily induce apoptosis via the intrinsic pathway of caspase activation, the extrinsic pathway also provides targets for lung cancer therapy. The TNF-related apoptosis-inducing ligand (TRAIL) and its receptors are of particular interest, as recombinant TRAIL exhibits antitumor activity in preclinical models, including lung cancer. Whereas TRAIL monotherapy has modest activity, it highly sensitizes xenografted lung cancers to cytotoxic drug-induced apoptosis *in vivo*. In agreement, the anti-TRAIL receptor antibody mapatumumab has no apparent antitumor activity in patients with advanced lung cancers; however, due to its favorable tolerability, further development in combination therapies seems warranted. In summary, further understanding of the role of apoptosis in lung cancer will likely improve therapeutic options and outcome of patients suffering from this devastating disease.

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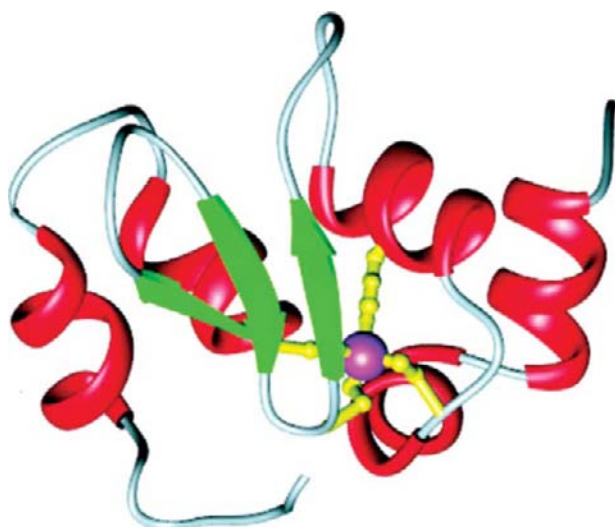


Plate 1. 3D structure of XIAP BIR3. See [Figure 2-1](#) for details.

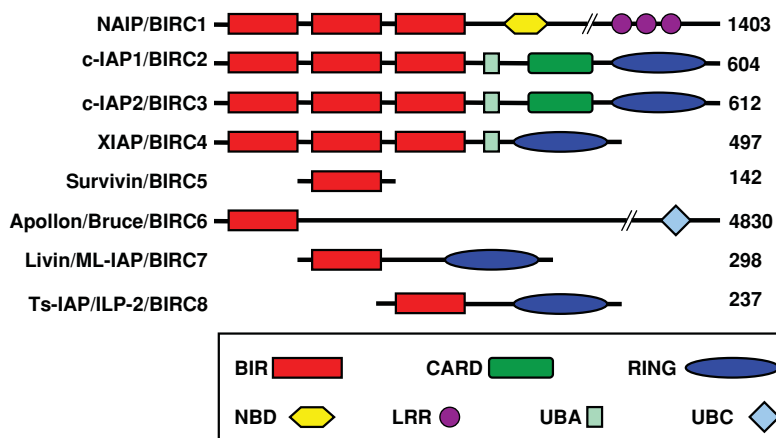


Plate 2. Domain organization of the human IAP protein family. See [Figure 2-2](#) for details.

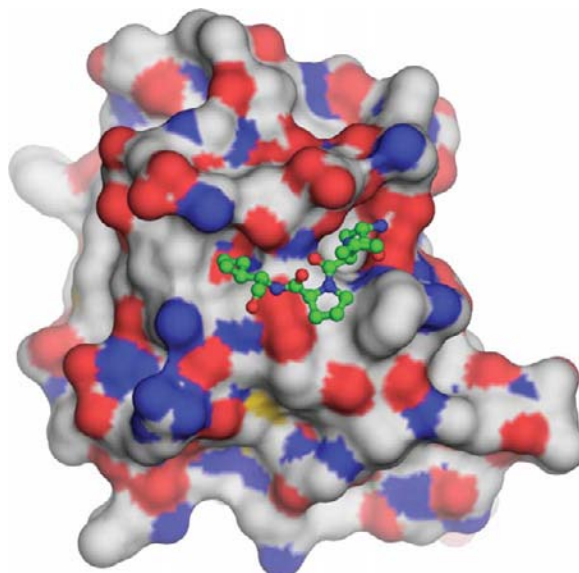


Plate 3. Structure of the XIAP BIR3 domain complexed with SMAC tetrapeptide. SMAC peptide bound to BIR3 of XIAP. The BIR3 domain of XIAP (shown as a space-filling model) complexed with the SMAC tetrapeptide, AVPI. See [Figure 2-4](#) for details.

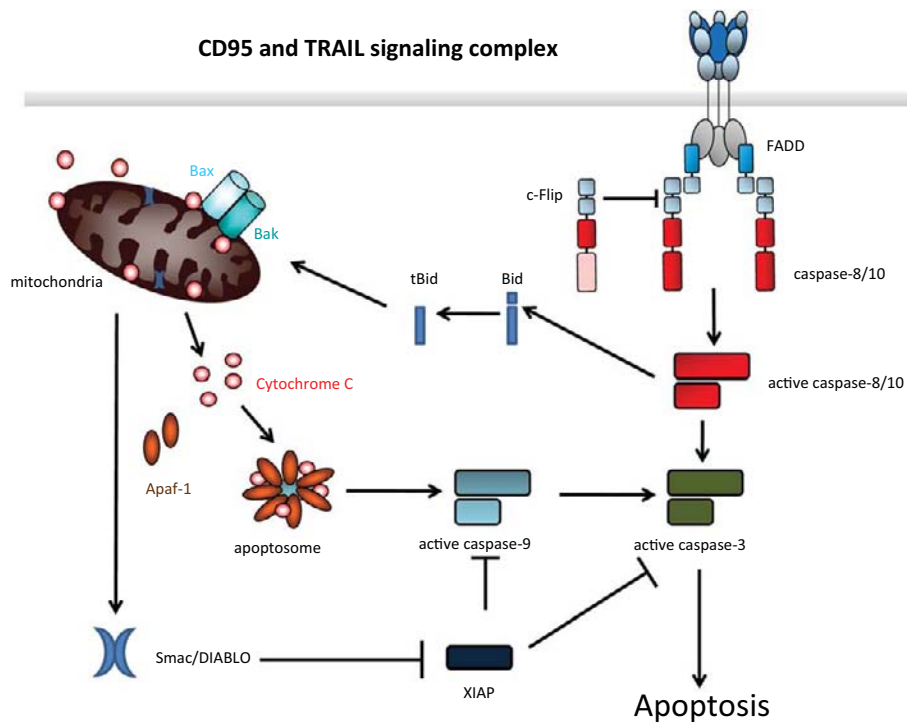


Plate 4. Schematic representation of apoptotic signaling by the CD95 and TRAIL systems. See [Figure 3-2](#) for details.

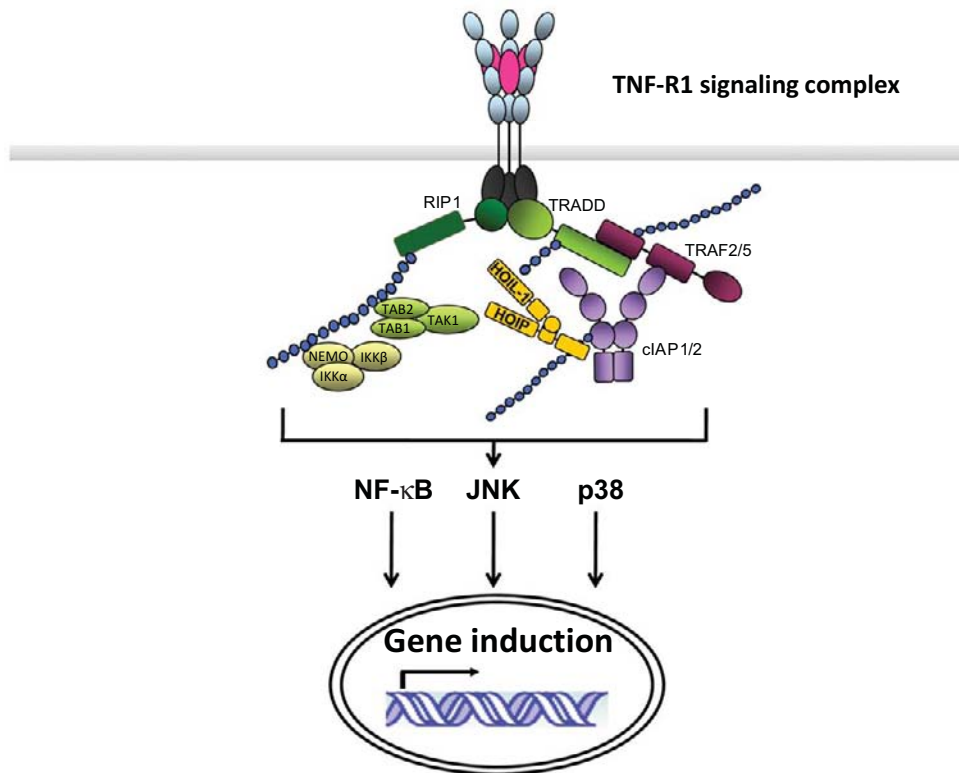


Plate 5. Schematic representation of immunostimulatory, pro-inflammatory signaling by the TNF-R and DR3 systems. Binding of TNF and TL1A to their respective receptor leads to receptor trimerisation and formation of a receptor signaling complex. See [Figure 3-3](#) for details.

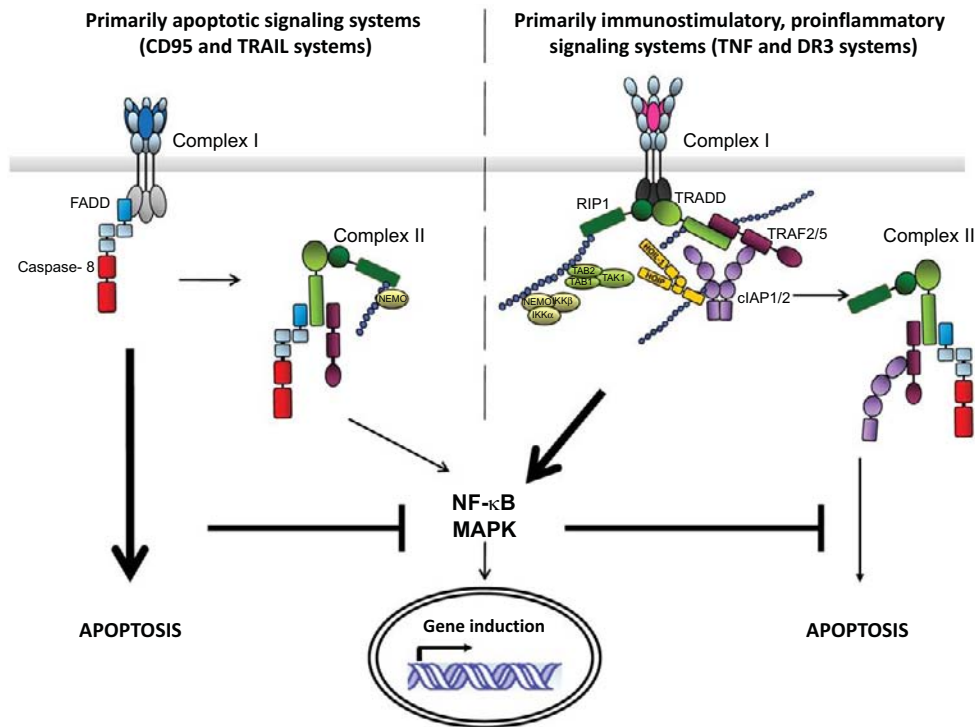


Plate 6. Complex I and complex II: spatial dissociation between proapoptotic and proinflammatory signaling in death receptor signal transduction. See [Figure 3-4](#) for details.

- - BIM or BID
- - sensitizer BH3-only proteins
- - cytochrome *c*
- Y - BCL-2 protein
- Y - BAX/BAK protein

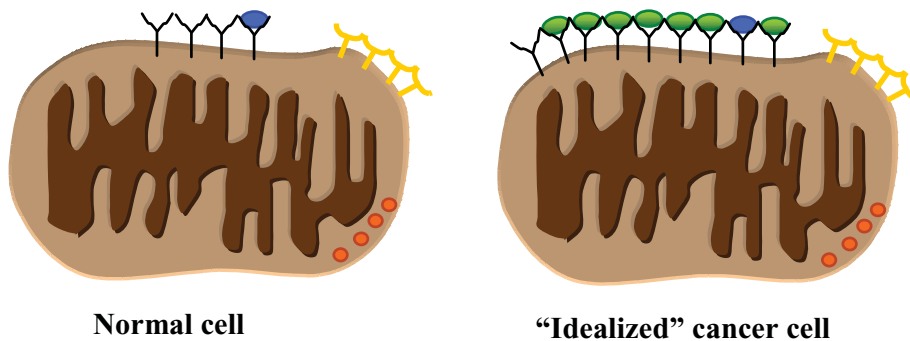


Plate 7. Cartoon representation of an unprimed mitochondrion versus a primed mitochondrion. See [Figure 5-3](#) for details.

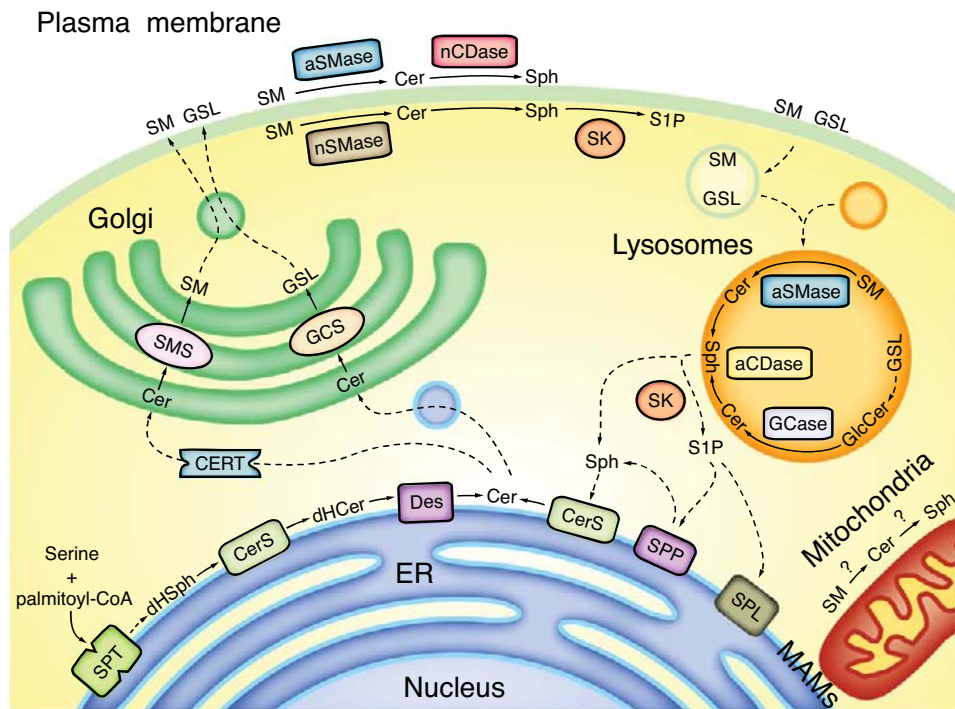


Plate 8. Compartmentalization of sphingolipid metabolism. See Figure 9-2 for details.

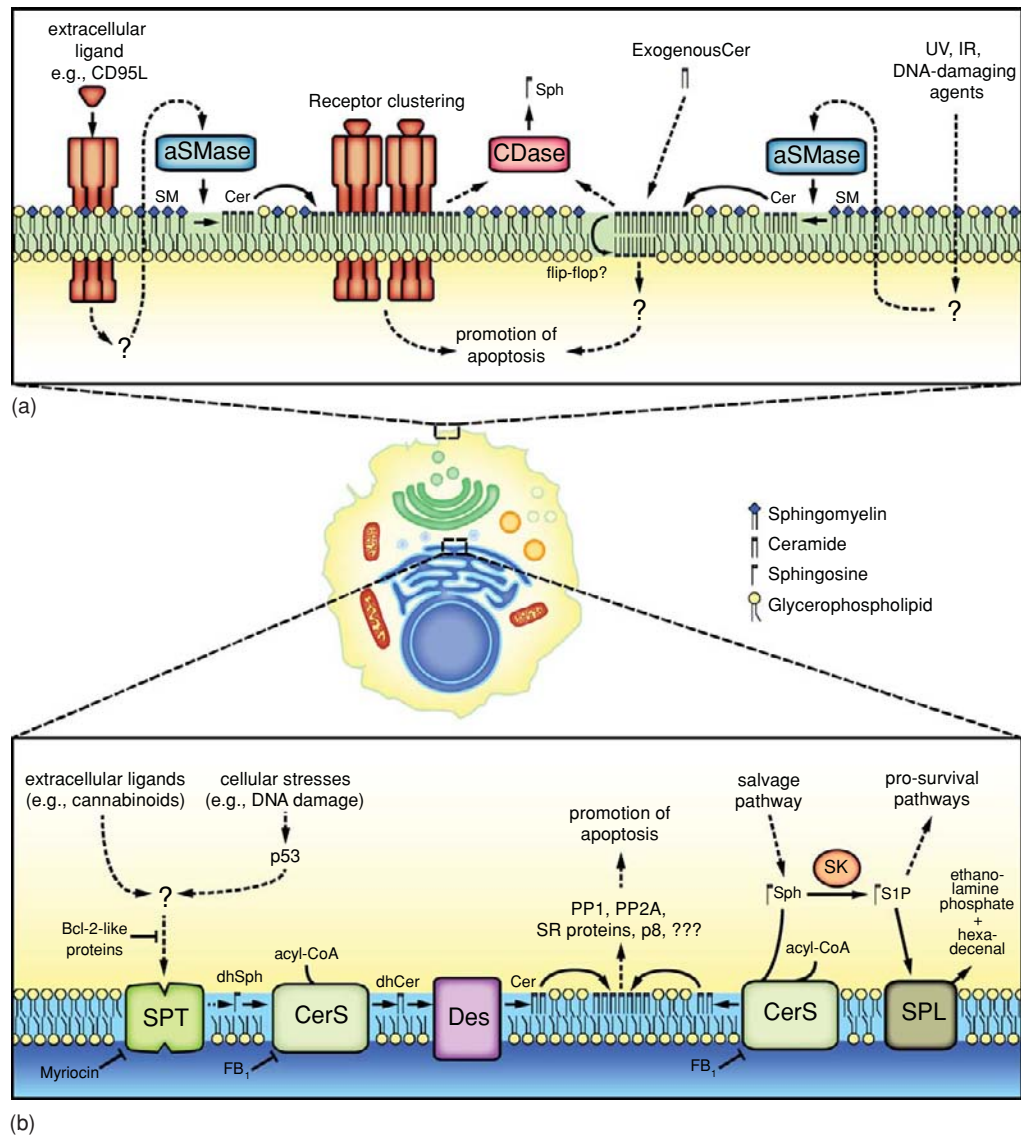


Plate 9. Summary of ceramide-mediated pathways. See [Figure 9-8](#) for details.

EFFECTOR CELL

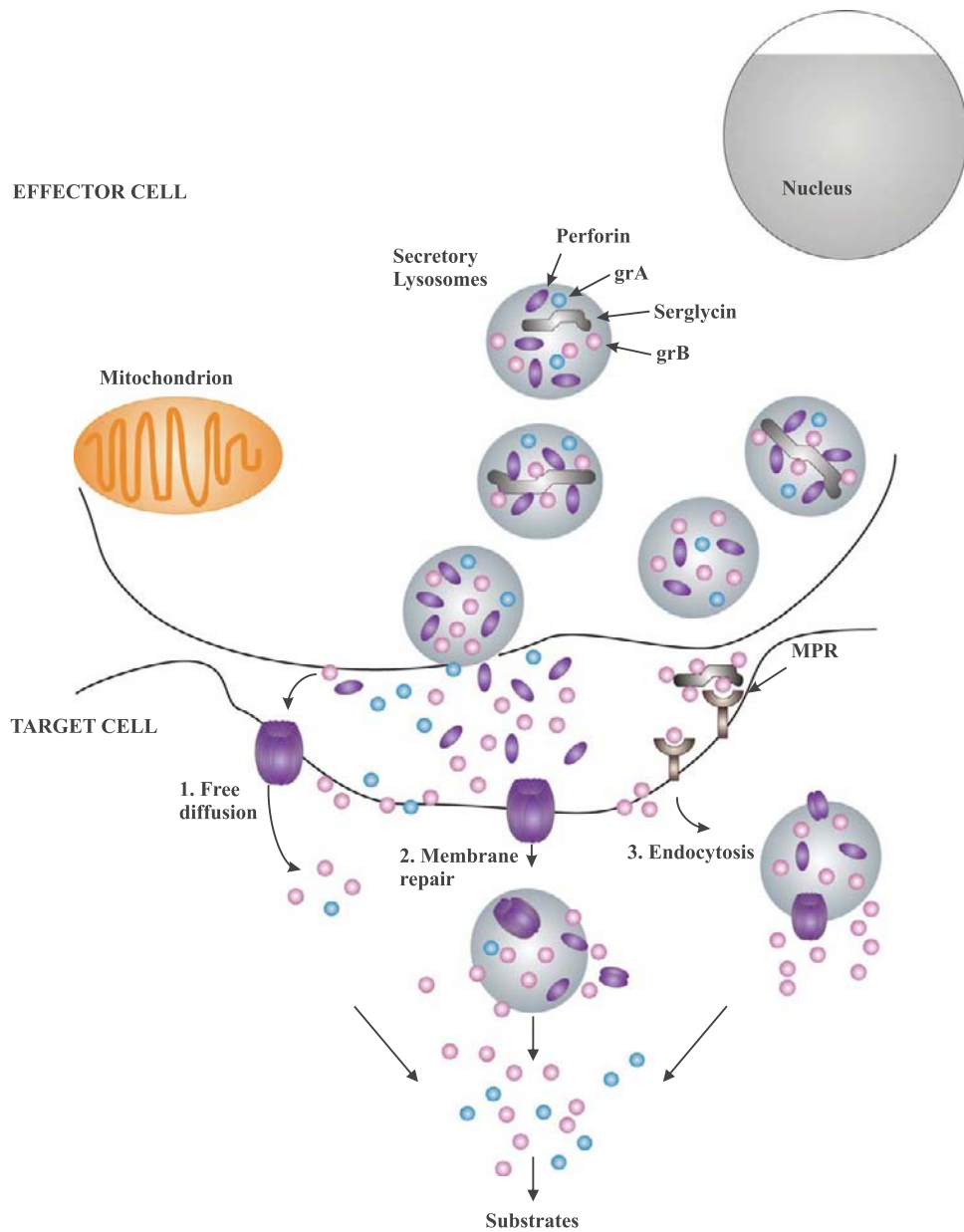


Plate 10. Several hypotheses have been proposed to explain how granzymes enter the target cell to mediate their cell death functions. See [Figure 10-1](#) for details.

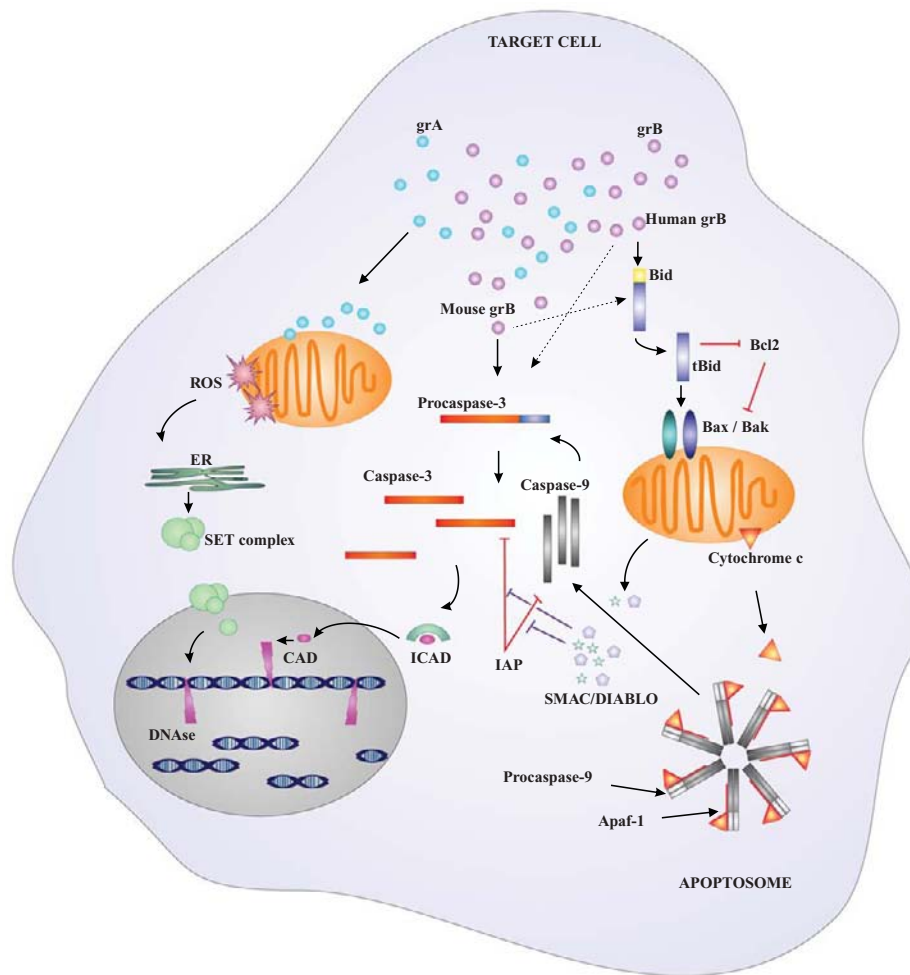


Plate 11. GrA and grB show different substrate specificities within the target cell. GrA induces the release of ROS from the mitochondrial inner membrane, which mediates the translocation of the SET complex from the ER to the nucleus. See [Figure 10-2](#) for details.

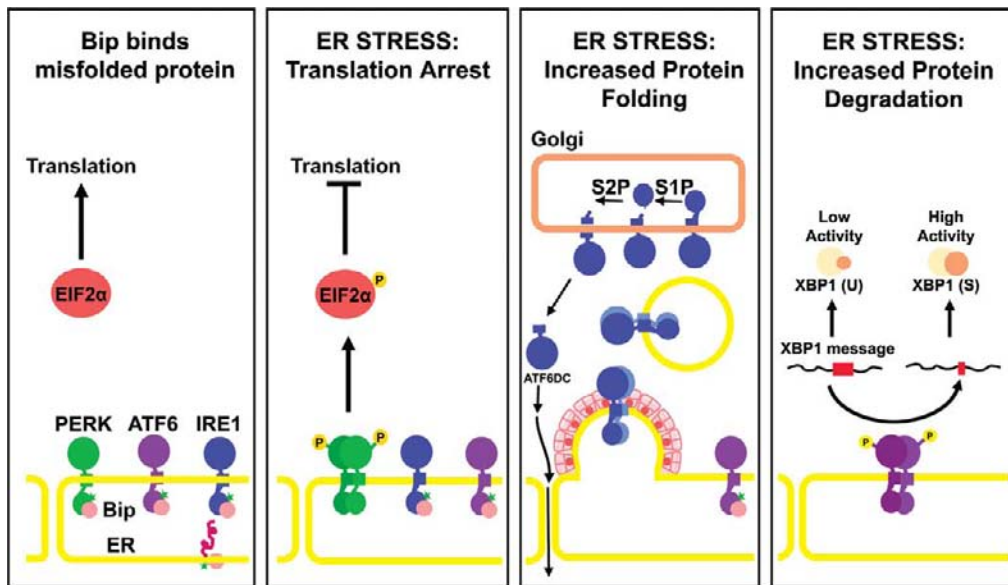


Plate 12. The unfolded protein response (UPR), a coordinated regulated response involving three sensor proteins: PERK (PKR-like ER kinase), ATF6 (activating transcription factor 6), and IRE1 (inositol requiring transmembrane kinase/endoribonuclease). See [Figure 12-1](#) for details.

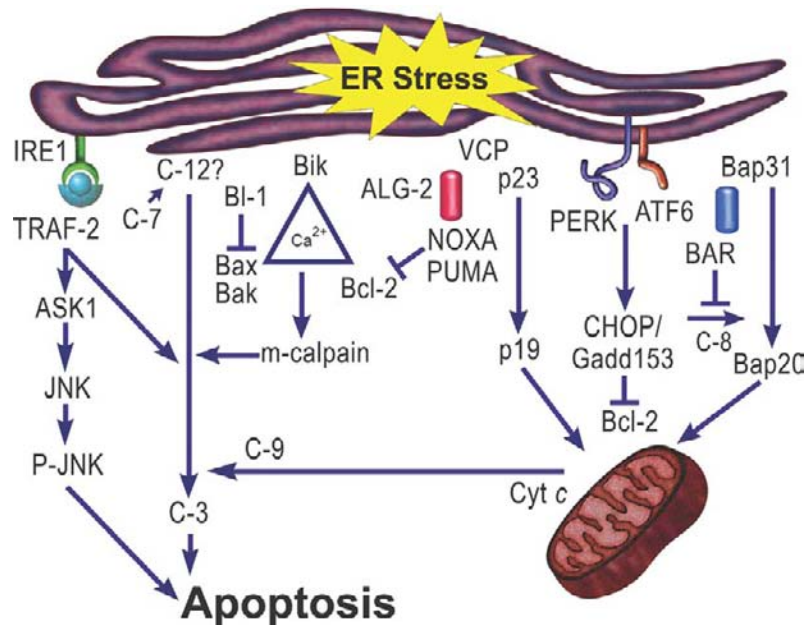


Plate 13. Proteins implicated in ER stress-pcd pathways. See [Figure 12-2](#) for details.

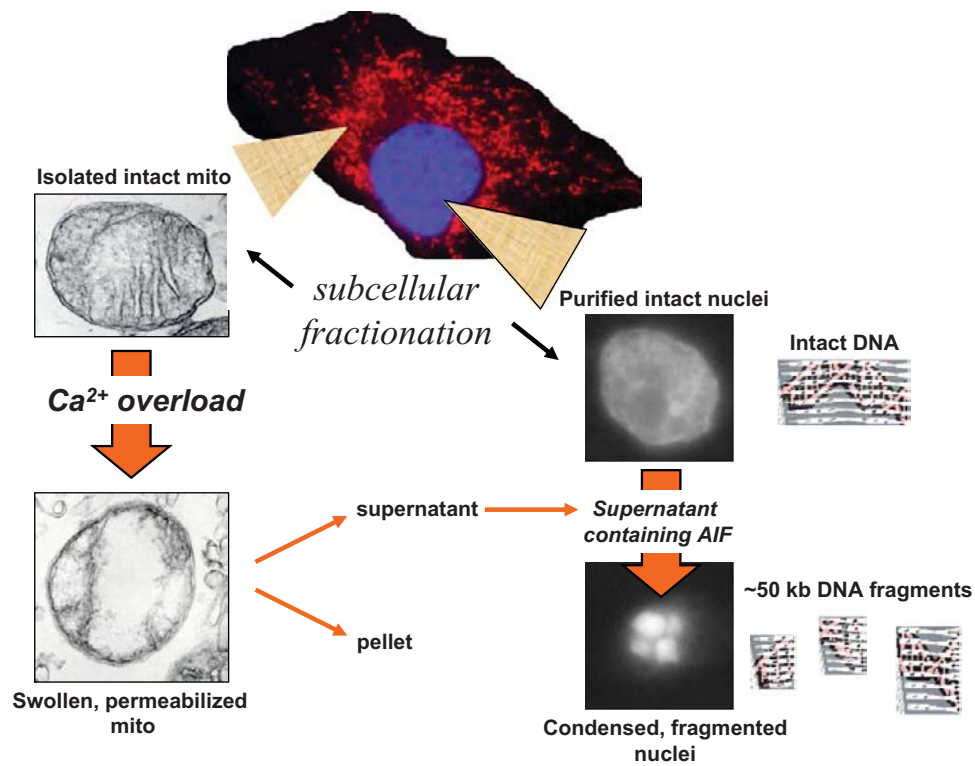


Plate 14. Identification of a caspase-independent mitochondrial apoptosis-inducing factor using an in vitro reconstitution assay with subcellular fractions. See Figure 14-4 for details.

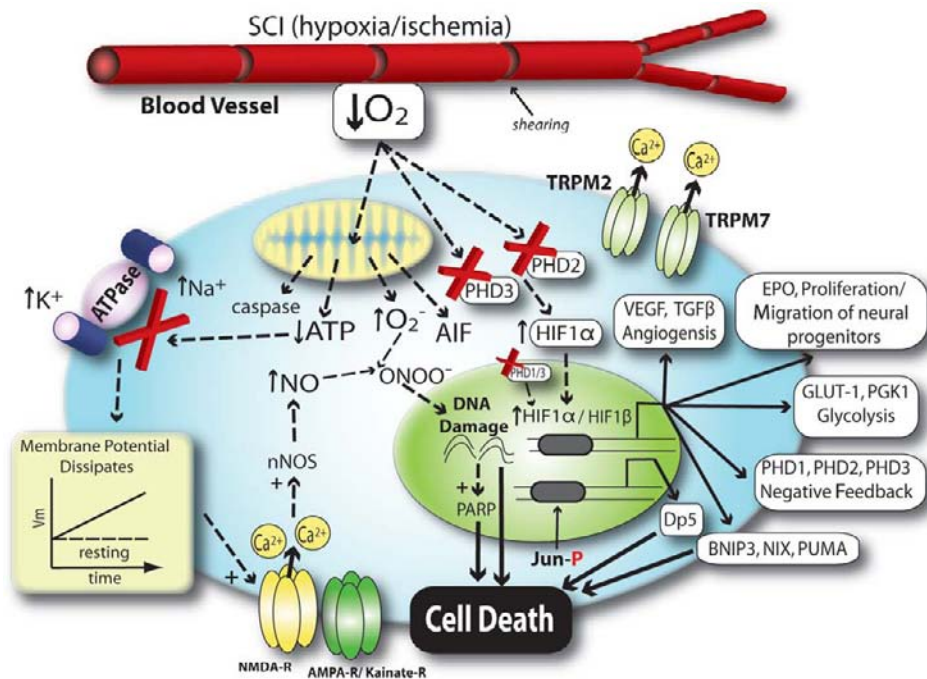


Plate 15. Extrinsic and intrinsic signals of cell death and survival after spinal cord injury. See Figure 15-1 for details.

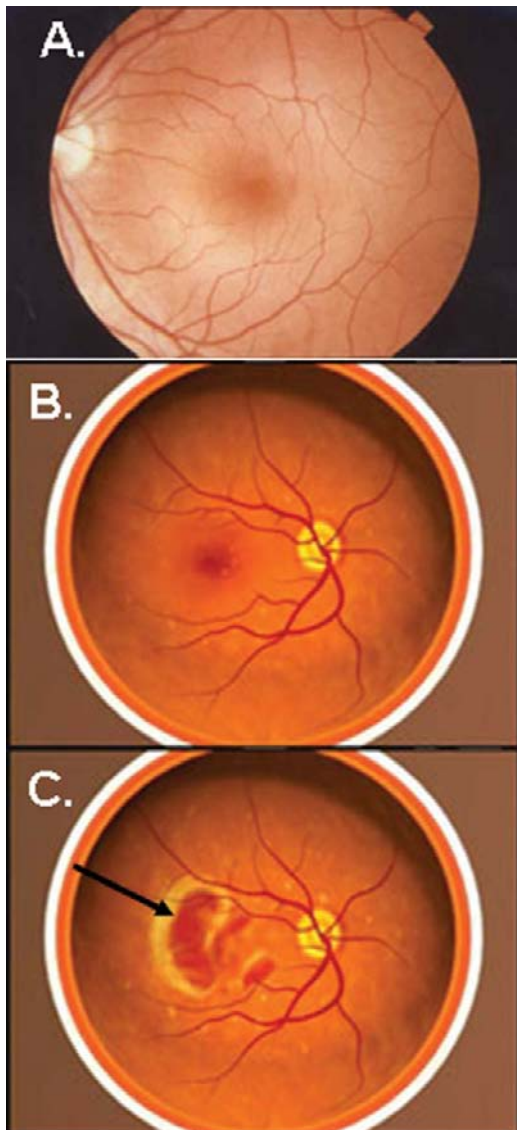


Plate 16. Retinal neovascularization in age-related macular degeneration (AMD). See [Figure 16-2](#) for details.

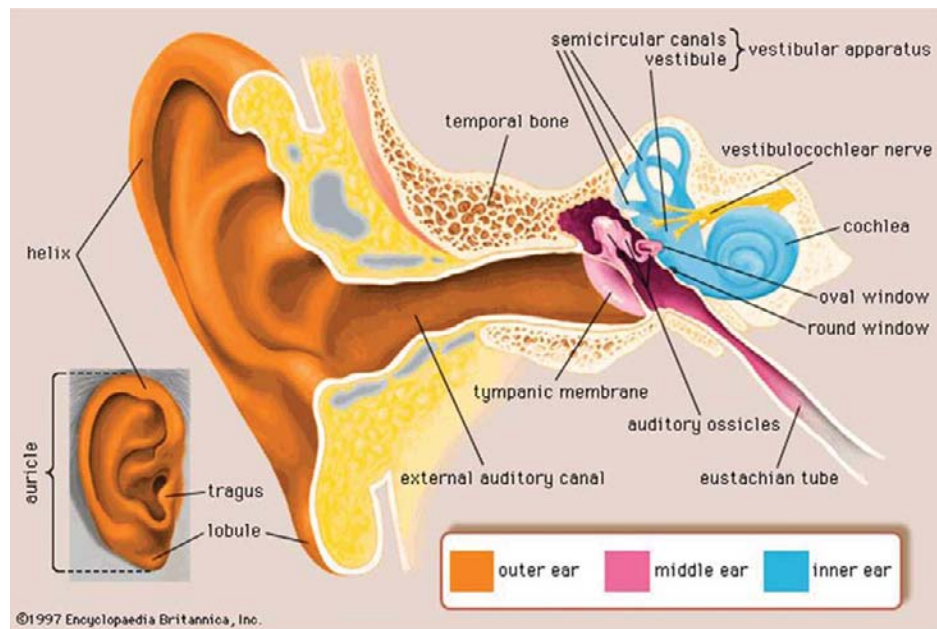


Plate 17. The ear. See Figure 17-1 for details.

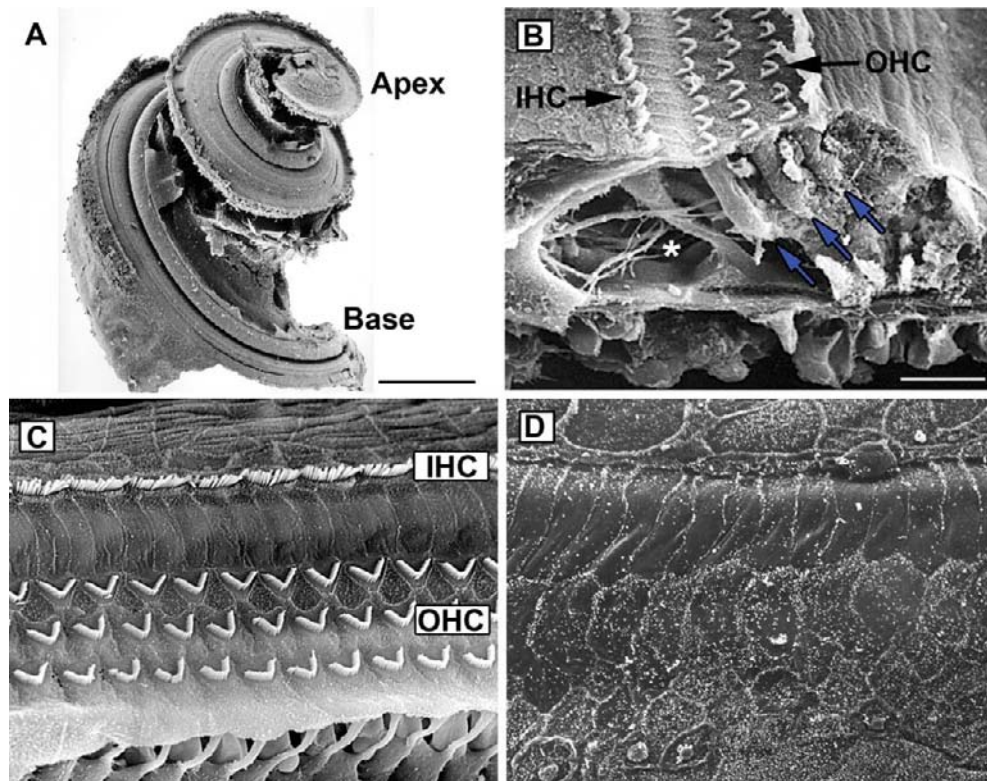


Plate 18. The cochlea. See [Figure 17-2](#) for details.

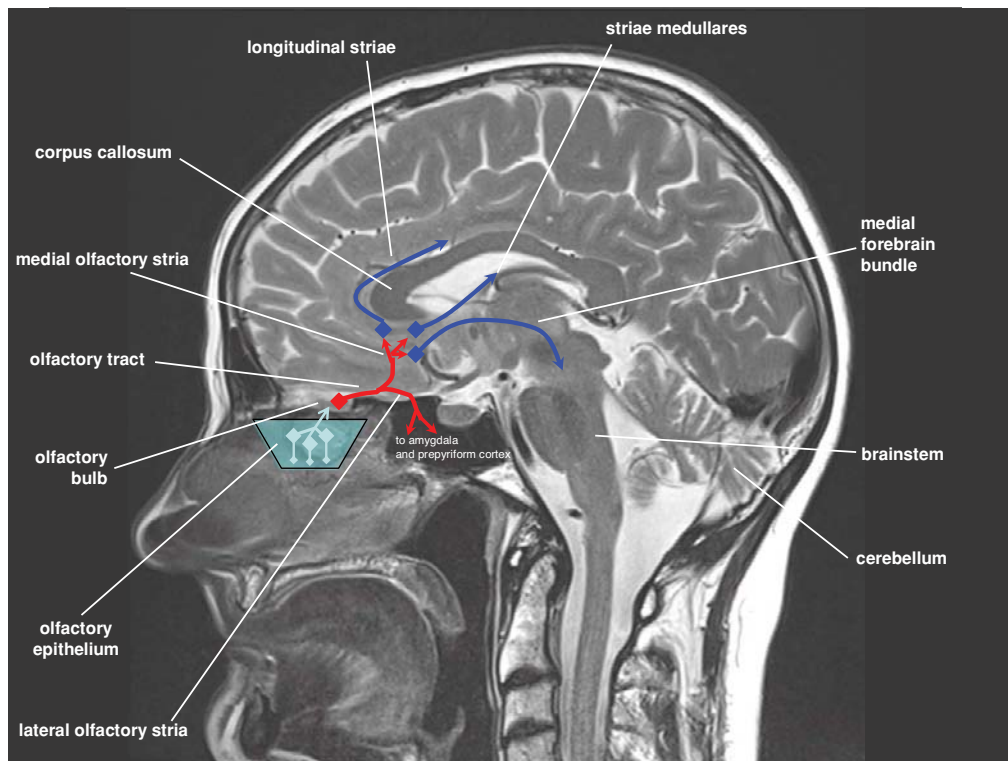


Plate 19. Gross anatomy. See [Figure 18-1](#) for details.

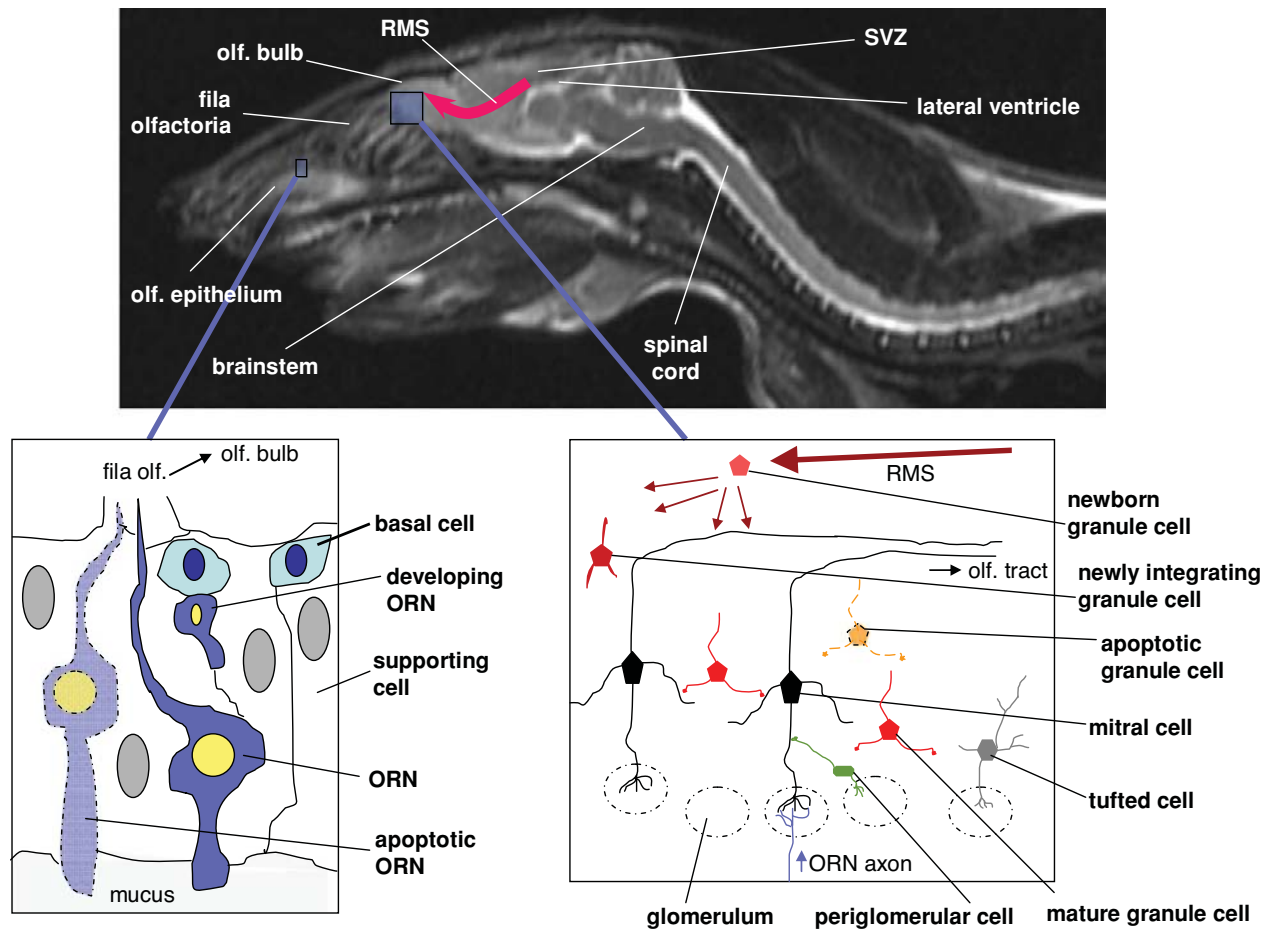


Plate 20. Olfactory life and death on a microscopic level. See [Figure 18-2](#) for details.

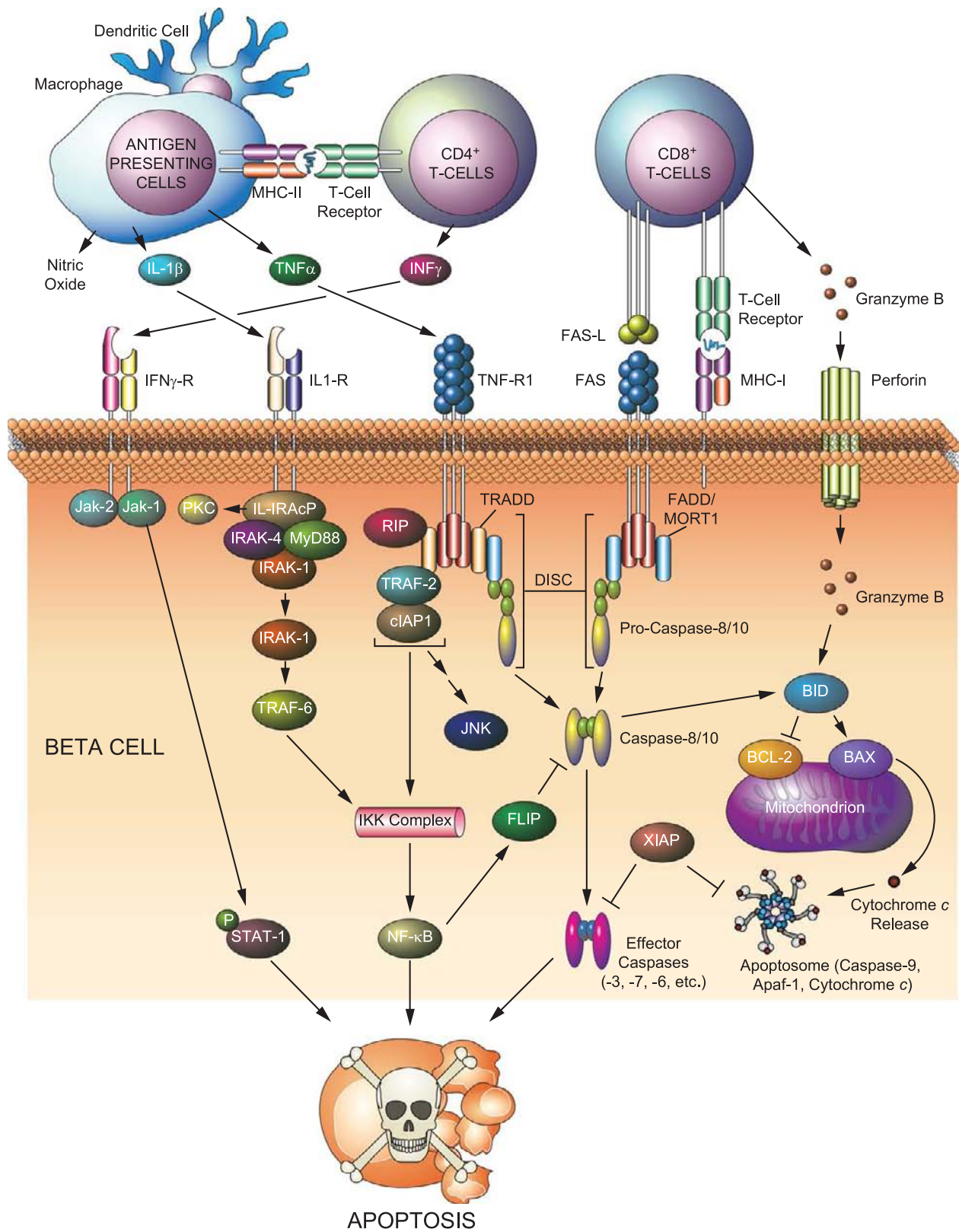


Plate 21. Signaling pathways leading to beta cell destruction in type 1 diabetes. See [Figure 19-1](#) for details.

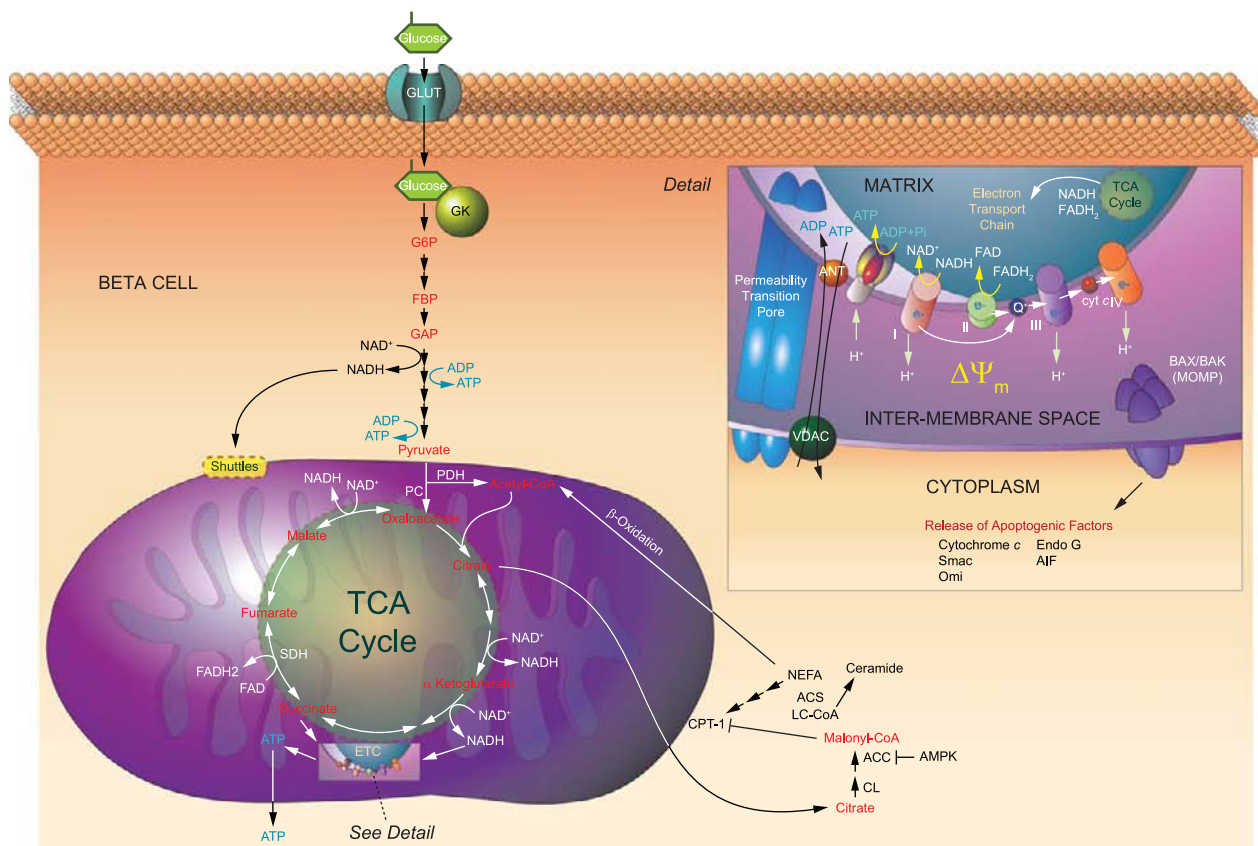
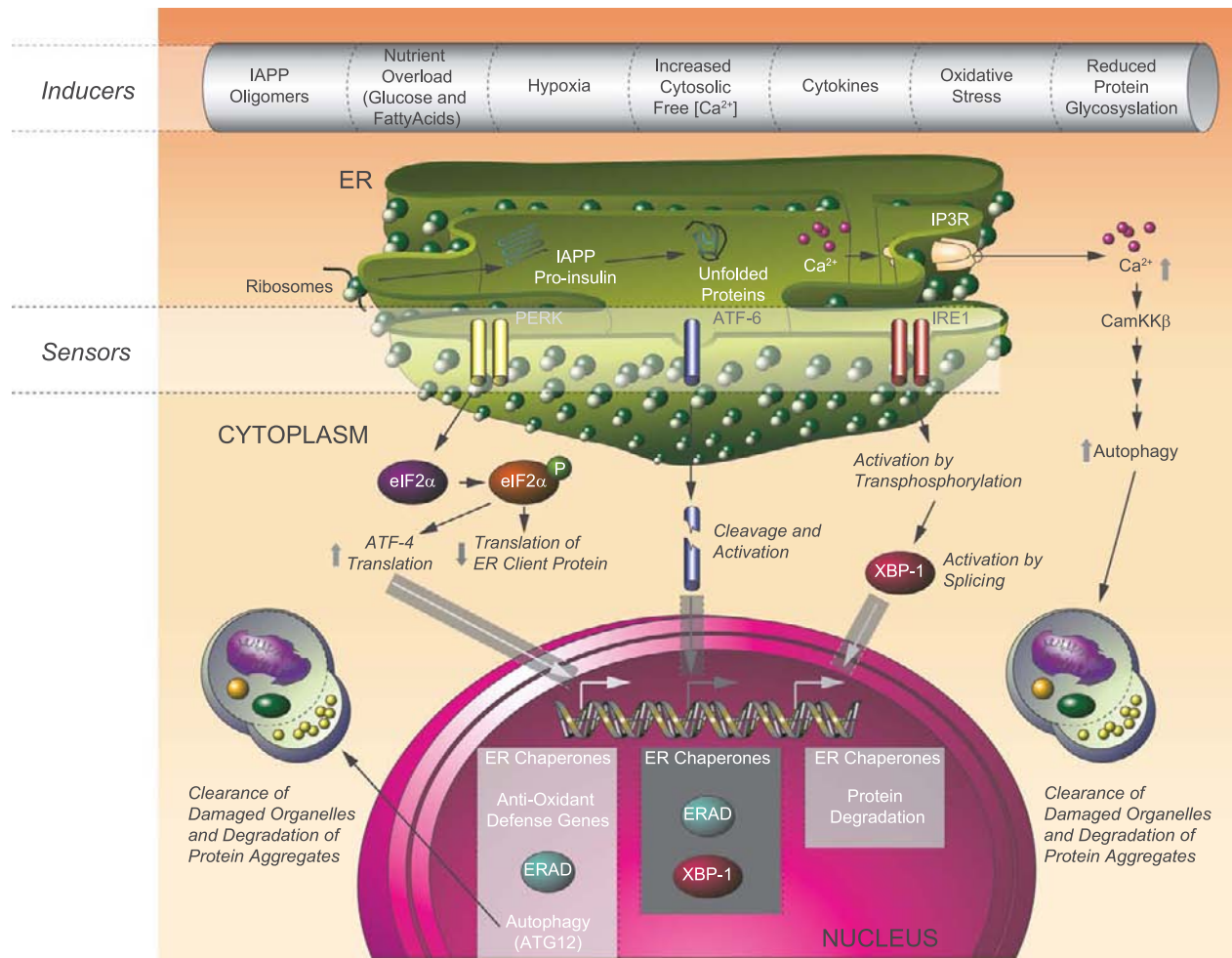
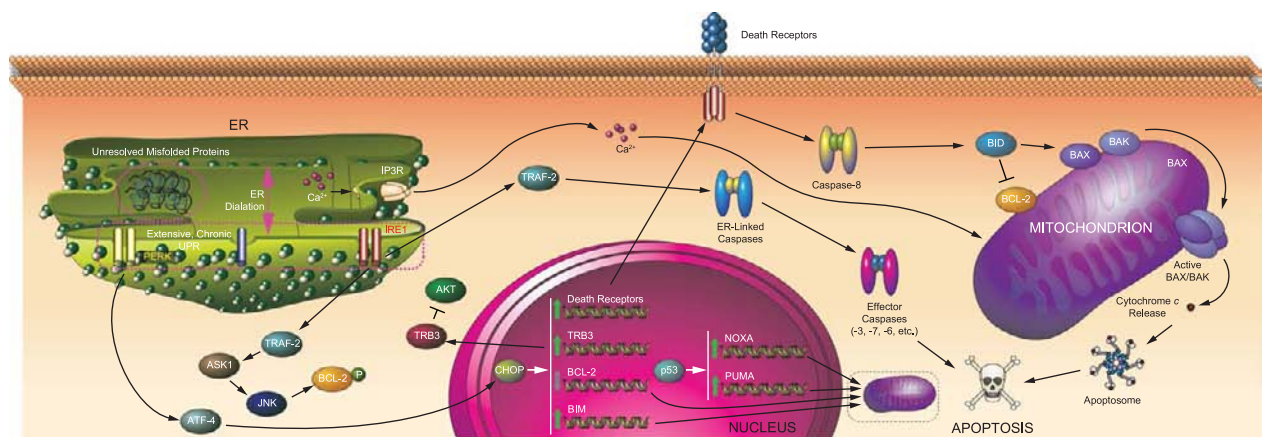


Plate 22. Shift in lipid partitioning associated with apoptosis in diabetic beta cells. See [Figure 19-2](#) for details.



(a)



(b)

Plate 23. Signaling pathways in response to unfolded proteins. See Figure 19-3 for details.

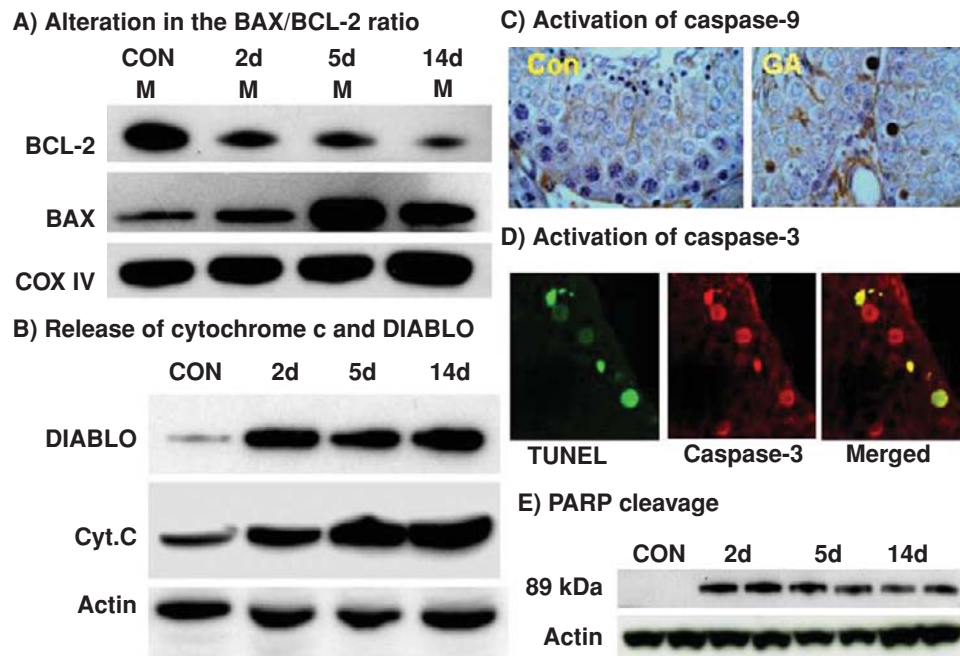


Plate 24. Hormonal deprivation results in activation of the intrinsic pathway signaling. See [Figure 25-3](#) for details.

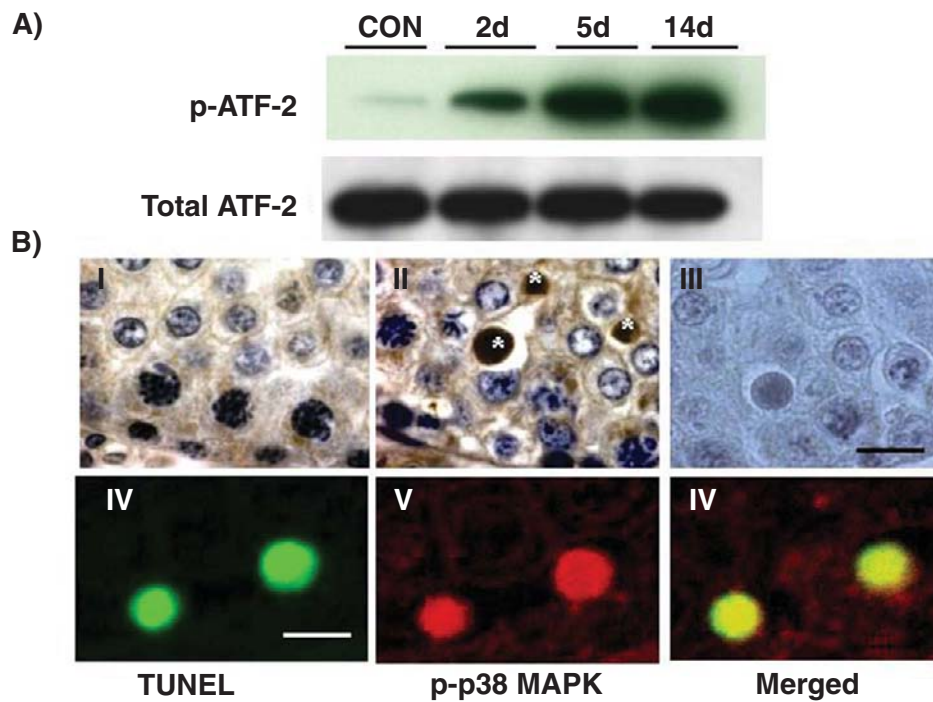


Plate 25. Activation of p38 MAPK in rat testes after GnRH-A treatment. See [Figure 25-4](#) for details.

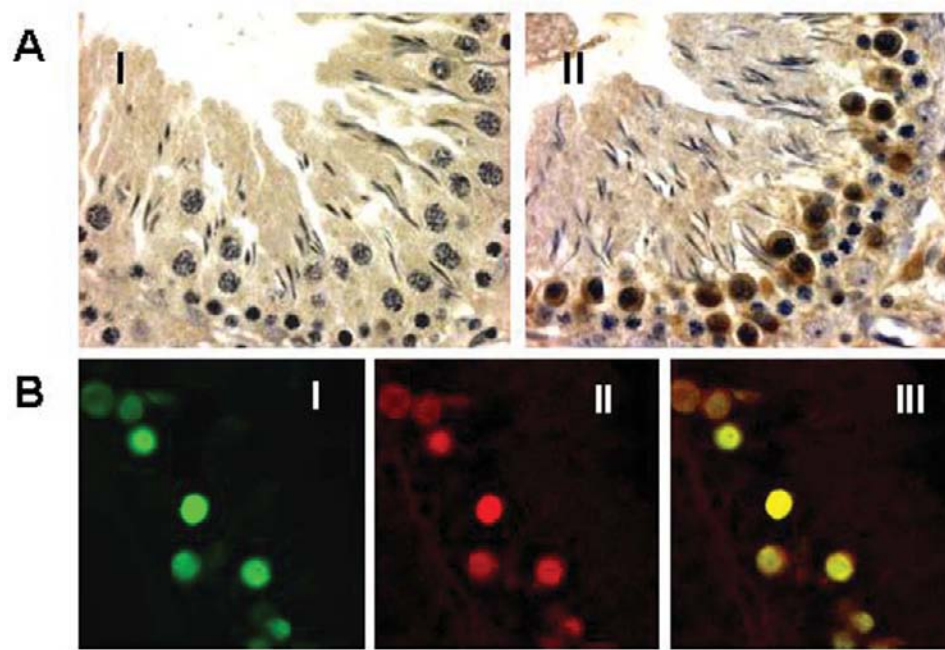


Plate 26. Testicular hyperthermia results in serine phosphorylation of BCL-2 in germ cells. See [Figure 25-5](#) for details.

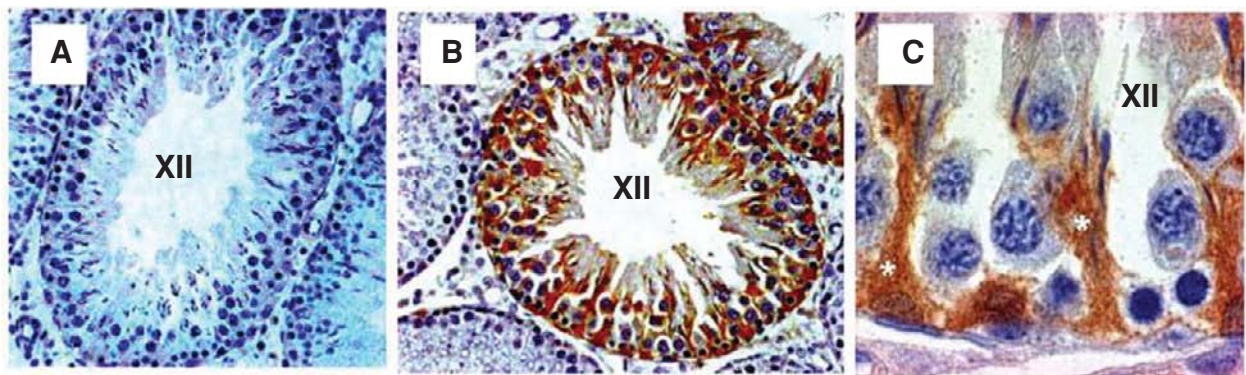


Plate 27. Activation of ERK in the Sertoli cells. See [Figure 25-6](#) for details.

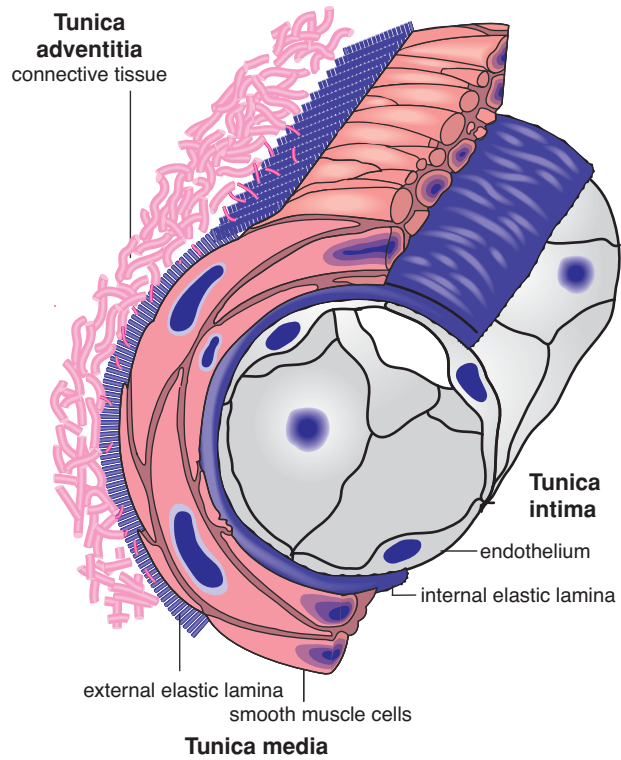


Plate 28. Normal human artery consists of three layers. See [Figure 26-1](#) for details.

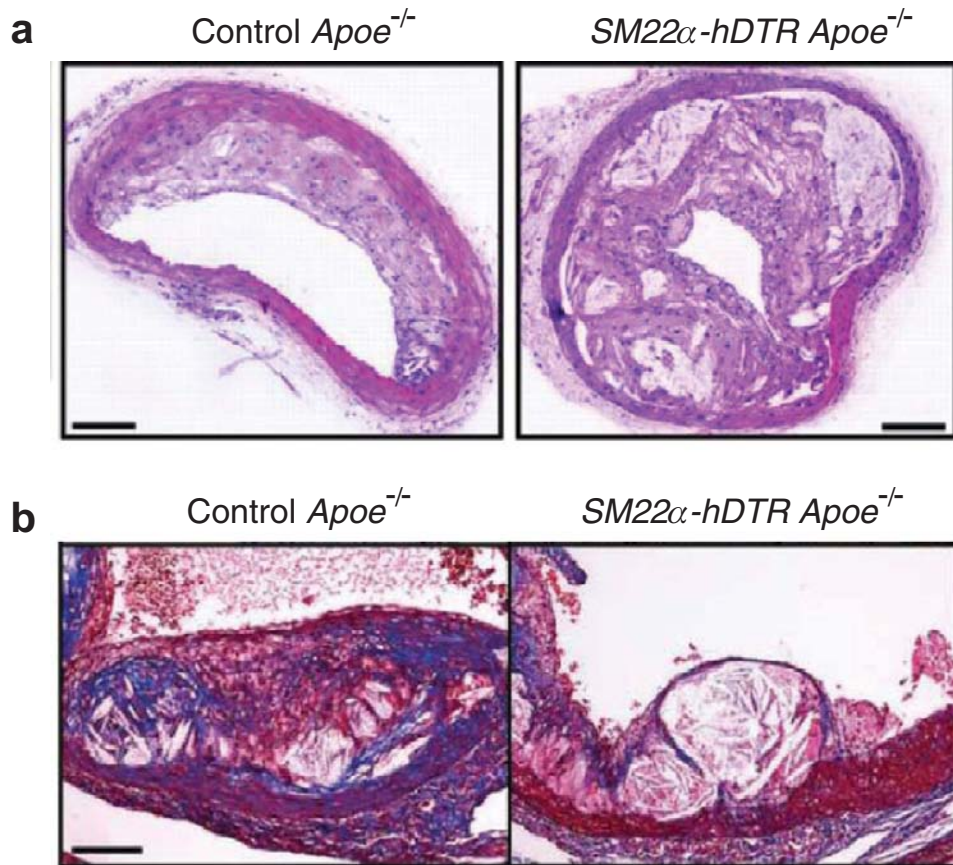


Plate 29. Vascular smooth muscle cell apoptosis accelerates atherosclerotic plaque progression and induces plaque vulnerability. See [Figure 26-3](#) for details.

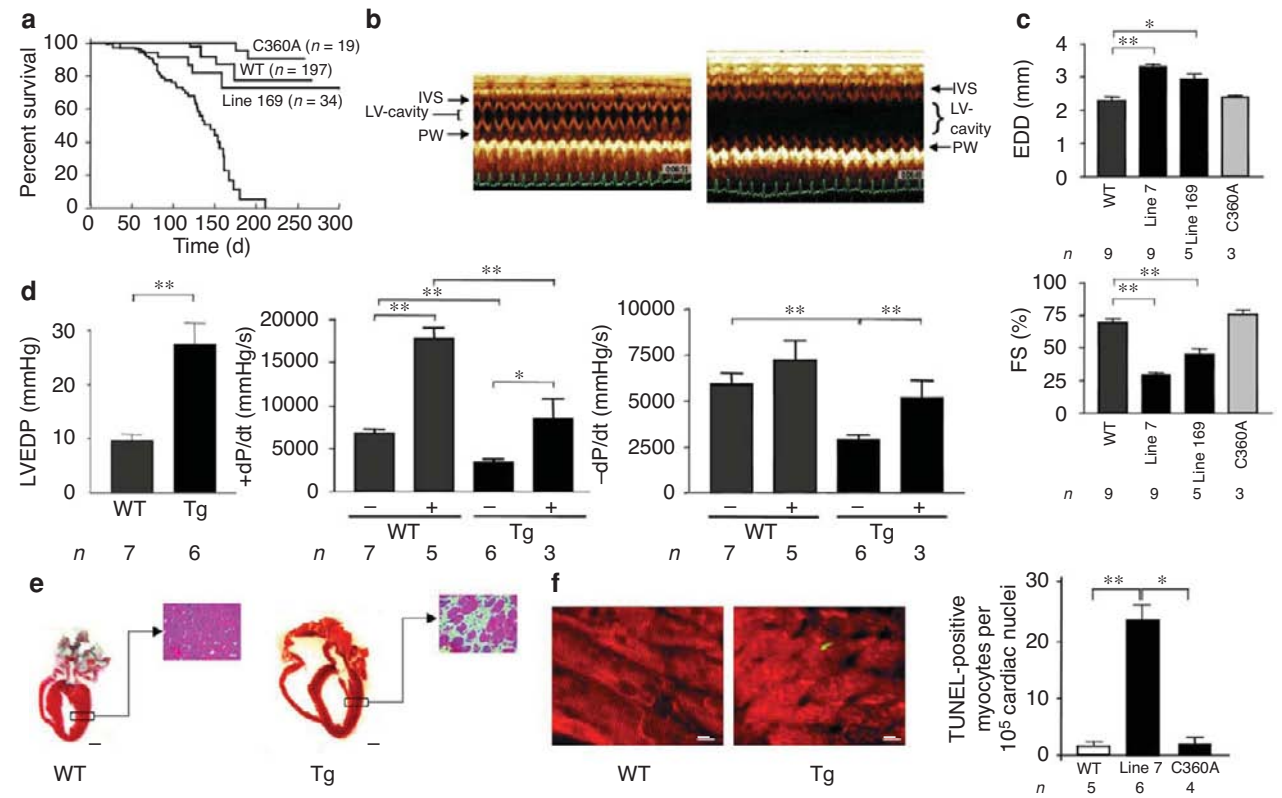


Plate 30. Modest, but elevated, rates of cardiac myocyte apoptosis are sufficient over time to induce lethal heart failure. See [Figure 26-4](#) for details.

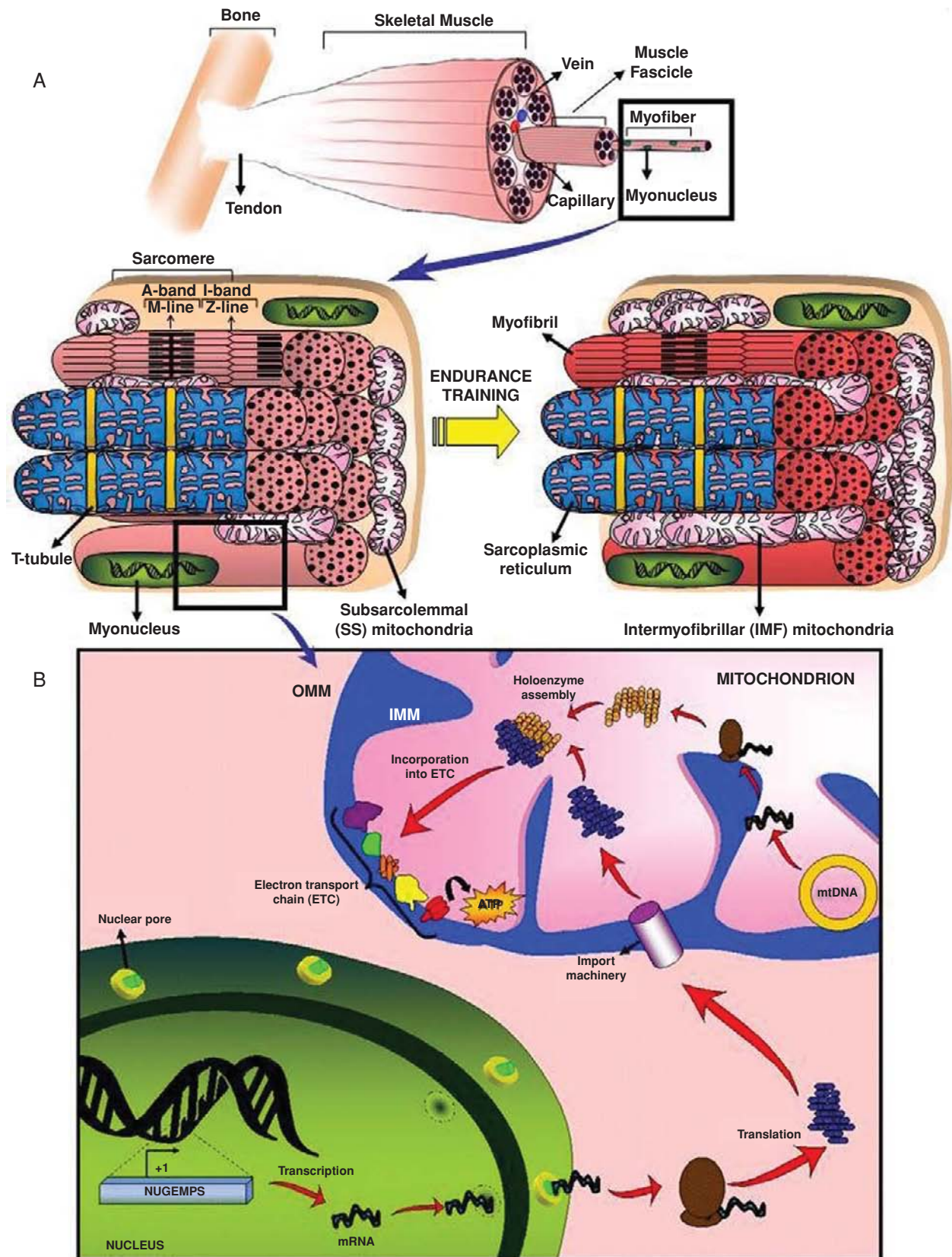


Plate 31. Unique morphology of skeletal muscle and exercise-induced mitochondrial biogenesis. See [Figure 27-1](#) for details.

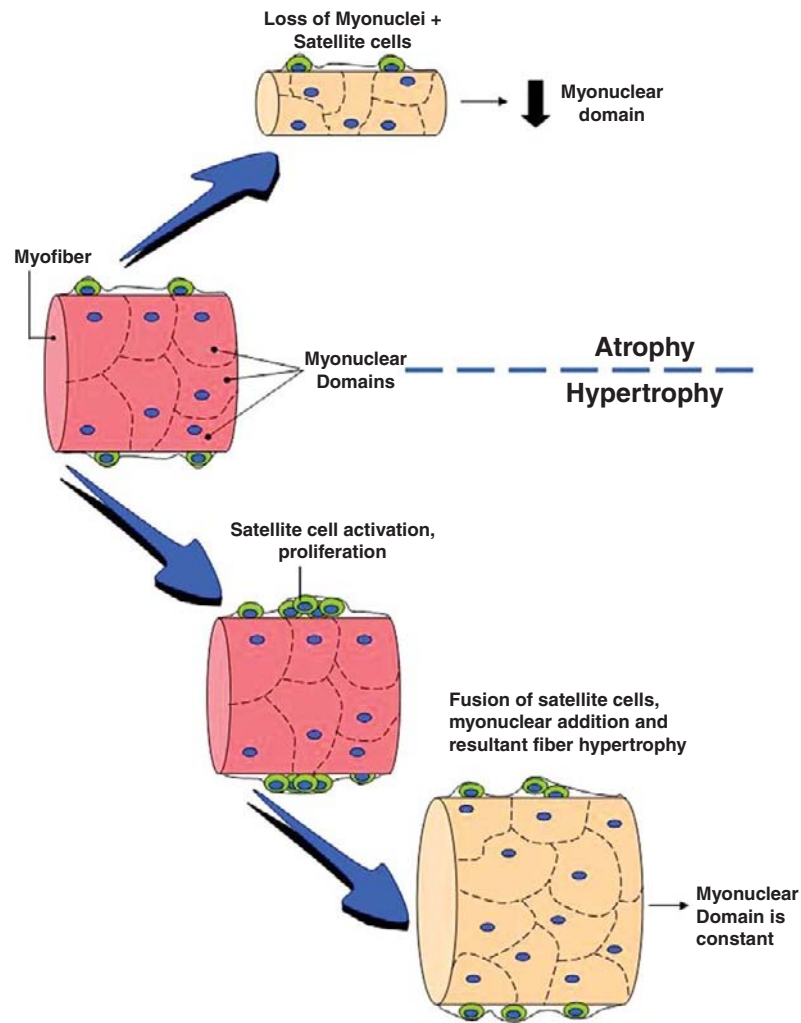


Plate 32. Myonuclear domains during muscle hypertrophy and atrophy. See [Figure 27-2](#) for details.

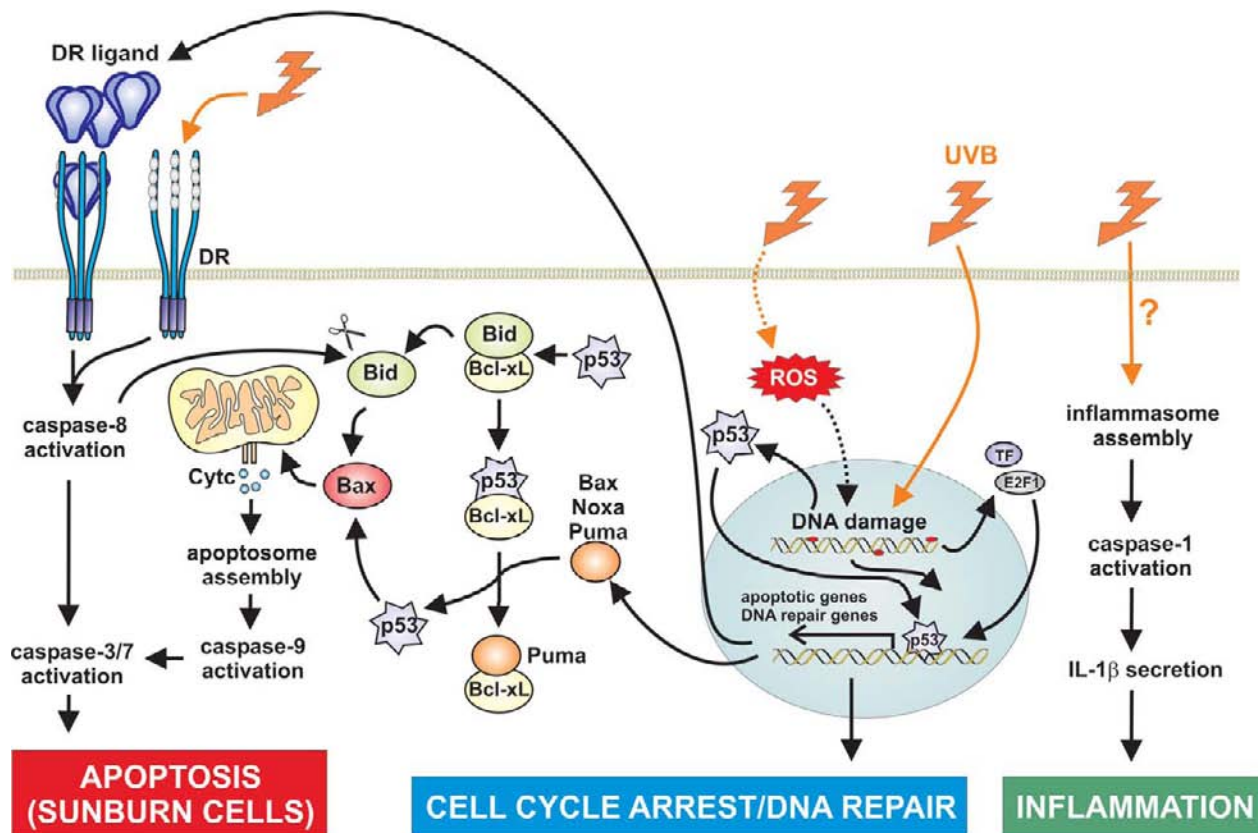


Plate 33. UVB signaling in keratinocytes. UVB can lead to different effects in keratinocytes, ranging from cell cycle arrest, apoptosis, and inflammasome activation. UVB radiation primarily damages nuclear DNA as a result of direct absorption and generates ROS that can induce oxidative damage to DNA and cellular proteins. See Figure 28-3 for details.

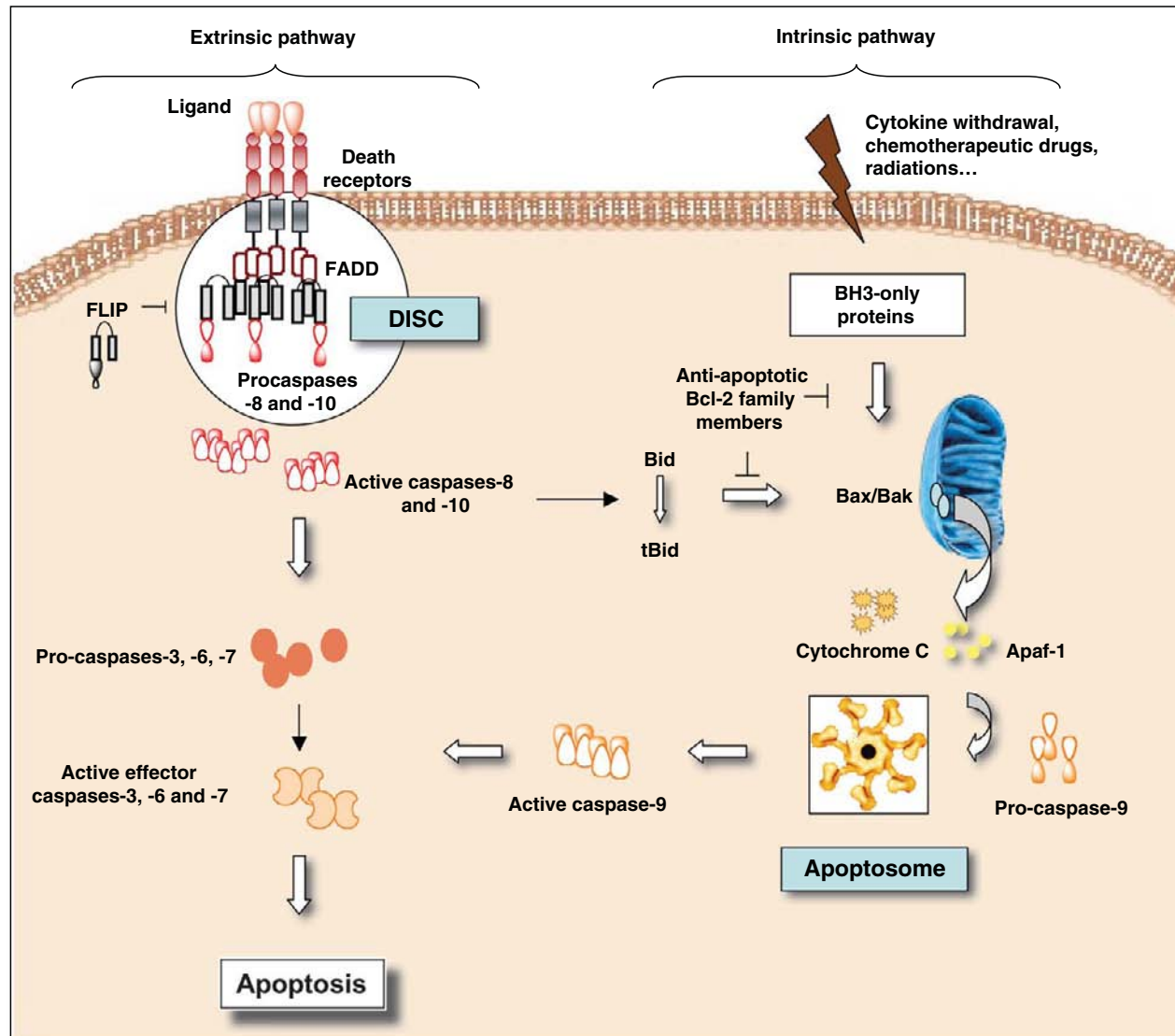


Plate 34. Two signaling pathways leading to apoptosis. See Figure 29-1 for details.

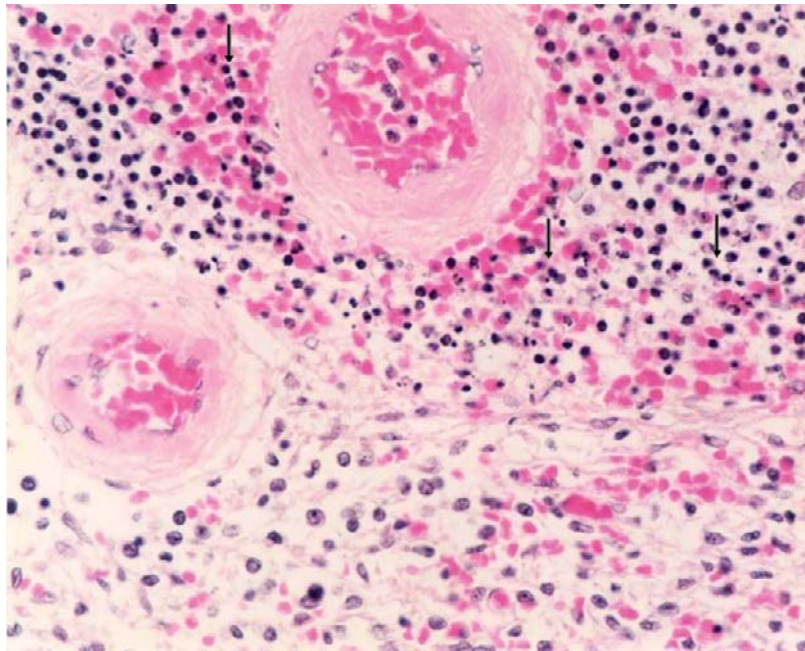


Plate 35. Lymphocyte apoptosis in spleen of septic patient. Hematoxylin and eosin staining of spleen of septic patient (400 × magnification). See [Figure 31-1](#) for details.

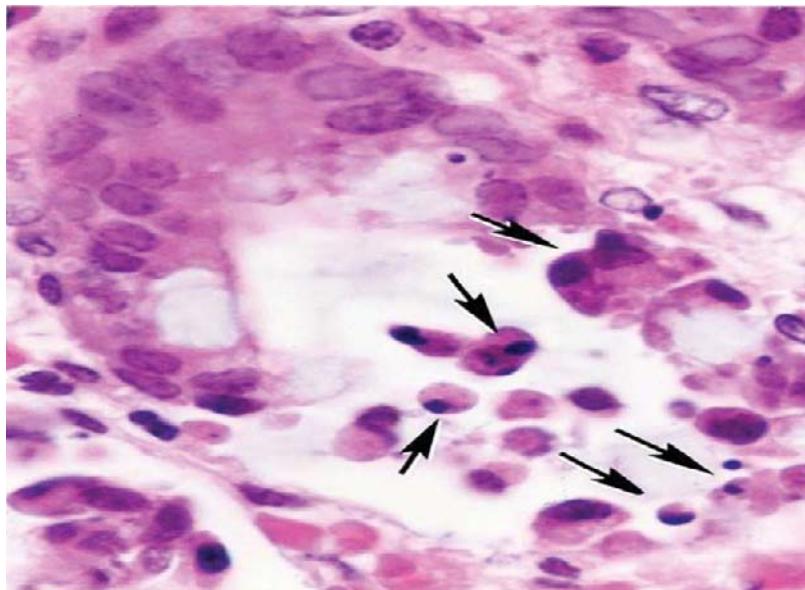
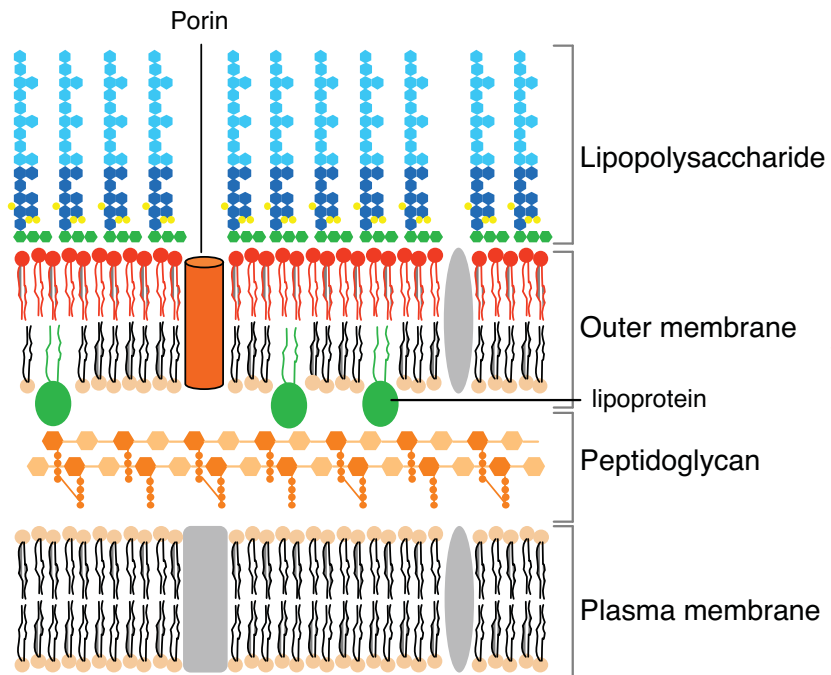


Plate 36. Colonic epithelial apoptosis in a septic patient. See [Figure 31-2](#) for details.

Gram-negative bacteria



Gram-positive bacteria

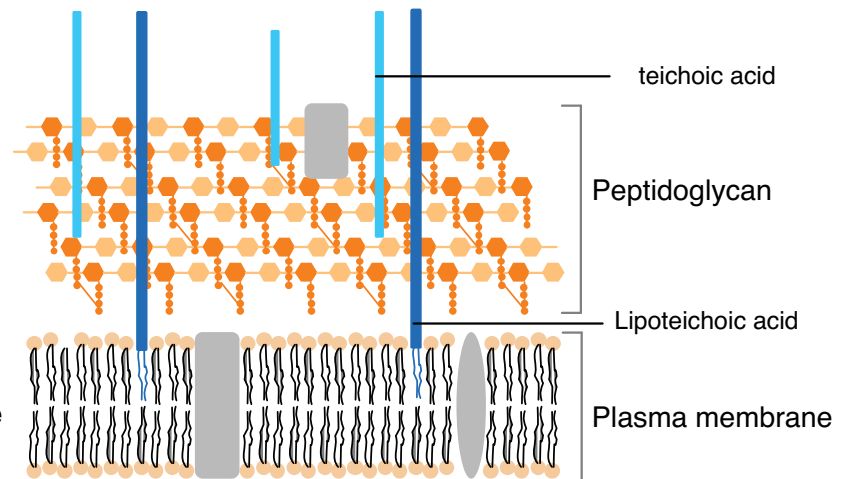


Plate 37. A representation of the cell wall from Gram-negative and Gram-positive bacteria. See [Figure 32-2](#) for details.

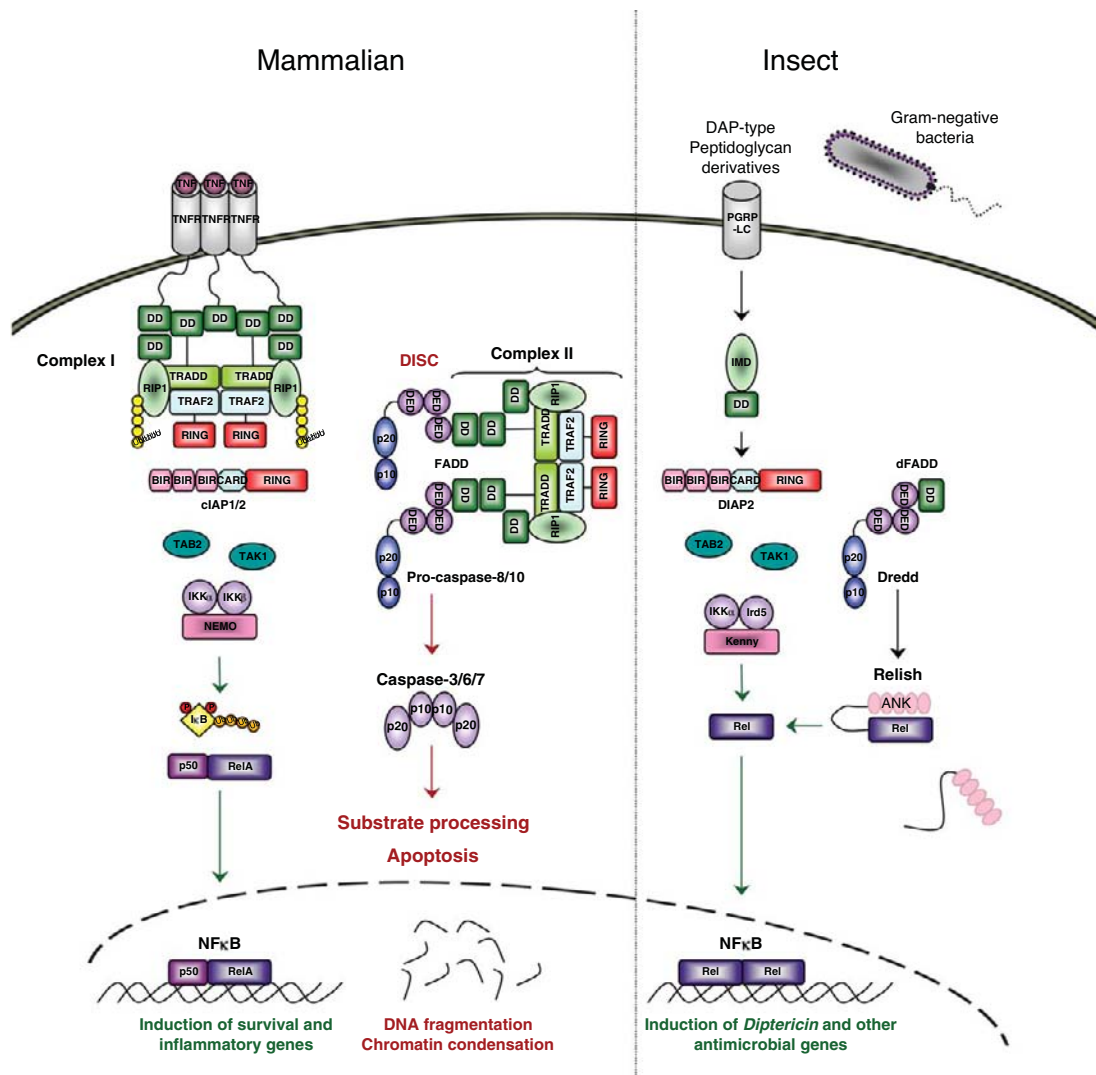


Plate 38. Schematic representation of the mammalian TNF and insect Imd pathways. See [Figure 32-6](#) for details.

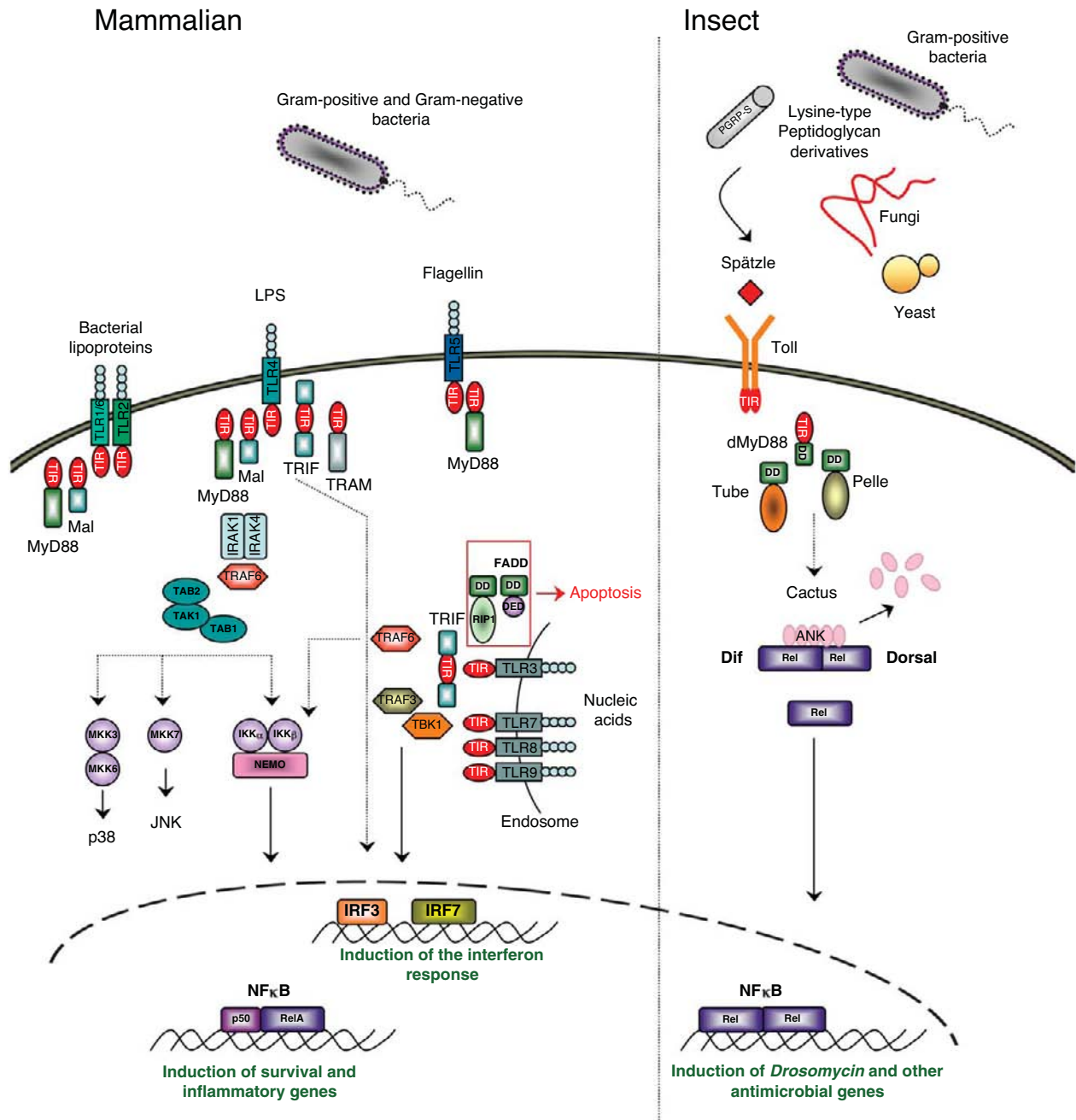


Plate 39. A schematic representation of the mammalian TLR and insect Toll pathways. See [Figure 32-7](#) for details.

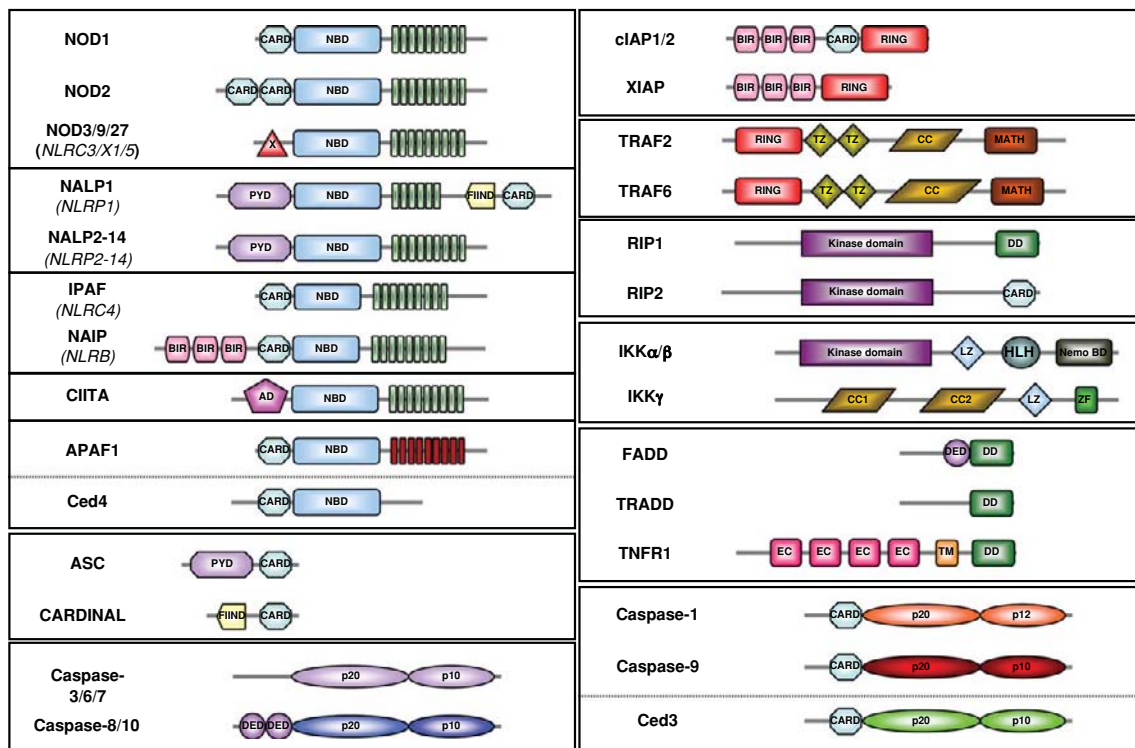


Plate 40. Effectors of inflammation. The domain structures are shown. See Figure 32-8 for details.

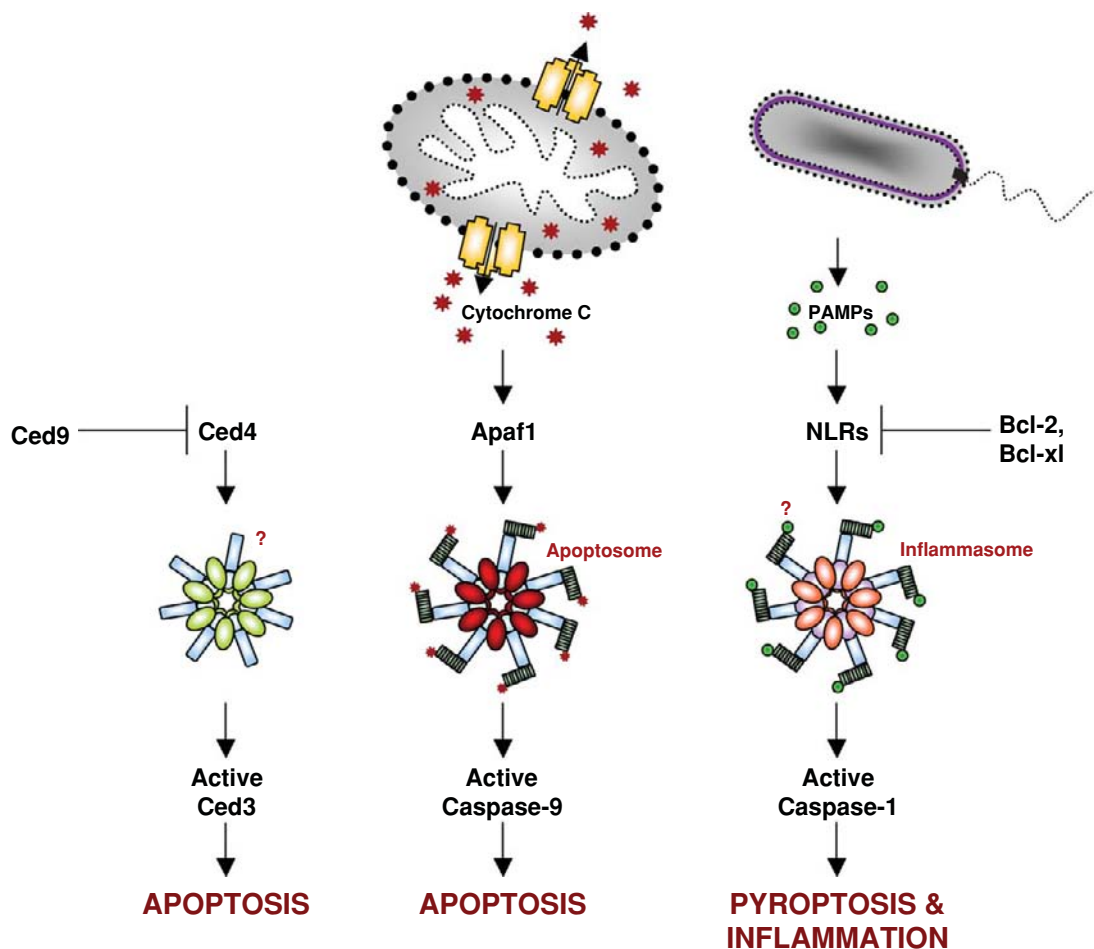


Plate 41. A parallel between mitochondrial apoptosis and NLR innate immunity pathways. See Figure 32-9 for details.

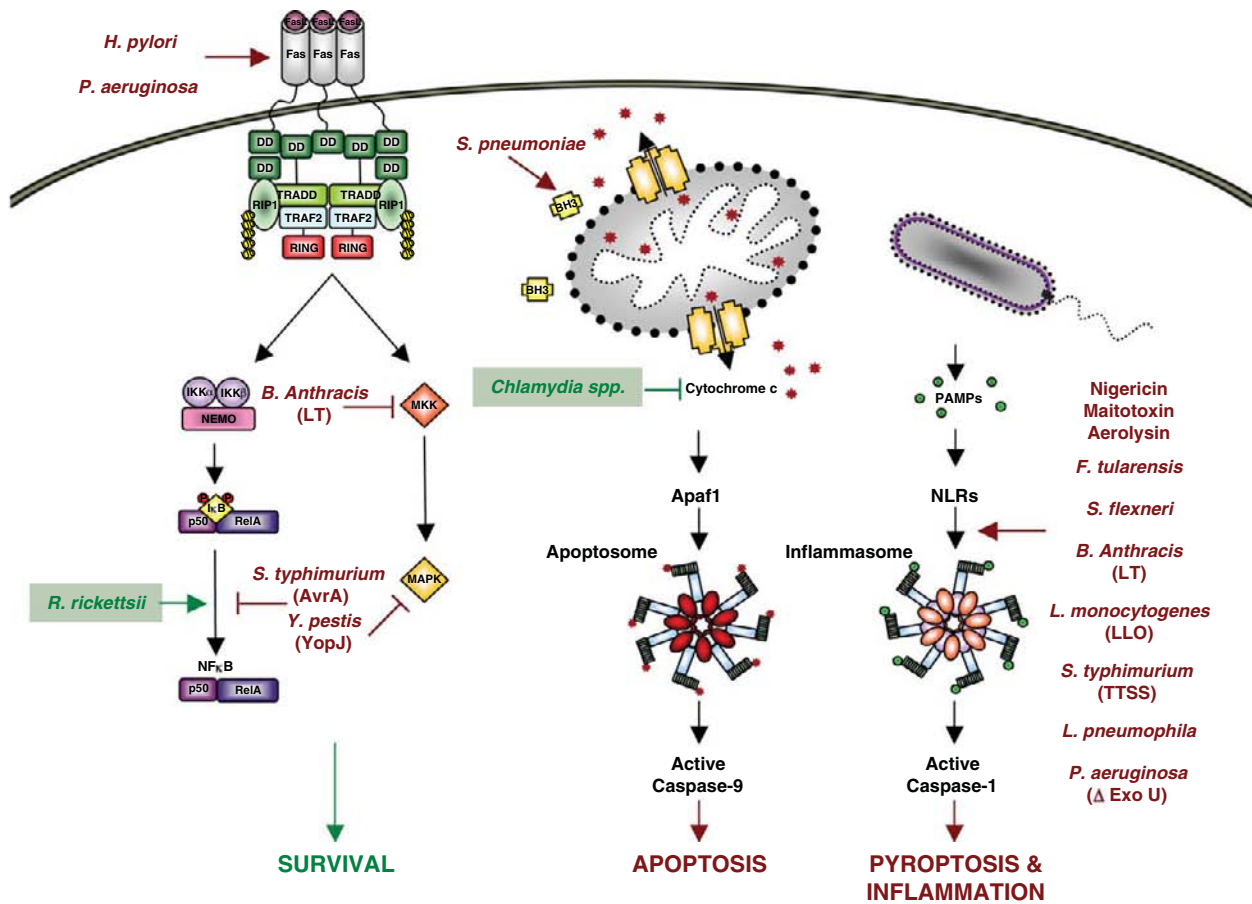


Plate 42. Modulation of cell survival/death pathways by microbial effectors. See Figure 32-10 for details.

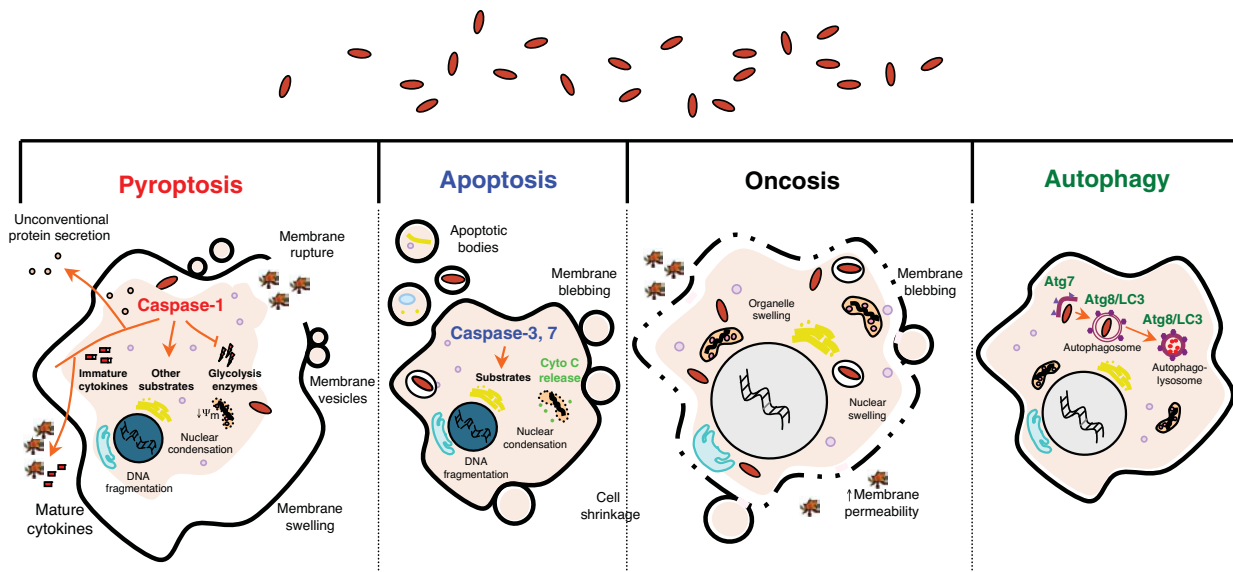


Plate 43. Pathogen-induced host cell death. See Figure 32-11 for details.

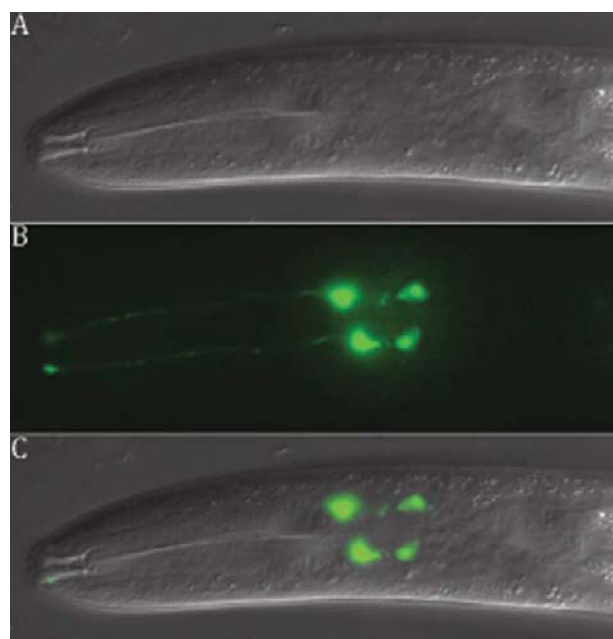


Plate 44. The four male-specific chemosensory (CEM) neurons located in the cephalic region of the animal undergo programmed cell death in hermaphrodites. See [Figure 34-3](#) for details.

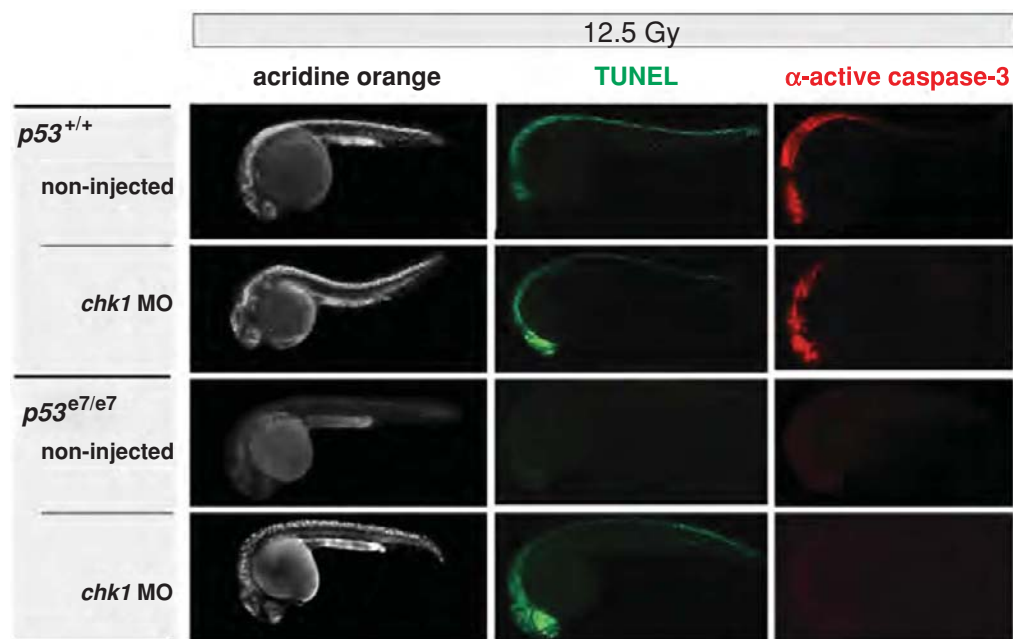


Plate 45. A rapid morpholino loss-of-function screen identifies *chk1* knockdown as a caspase-3-independent radiation sensitizer in *p53* mutant embryos. See [Figure 36-3](#) for details.

Regions of developmental cell death

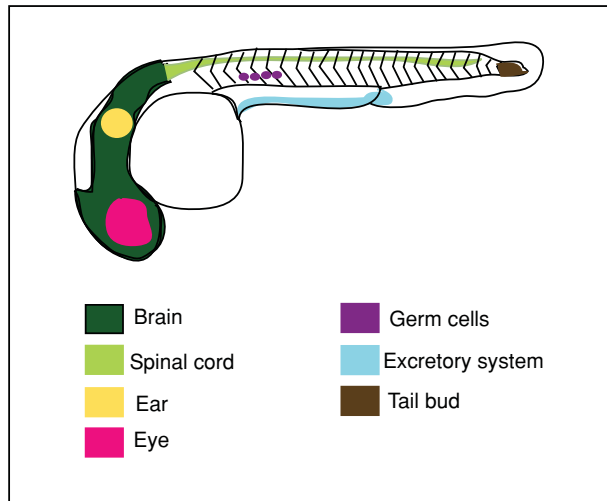


Plate 46. Cell death zones in developing zebrafish embryos. See [Figure 36-5](#) for details.

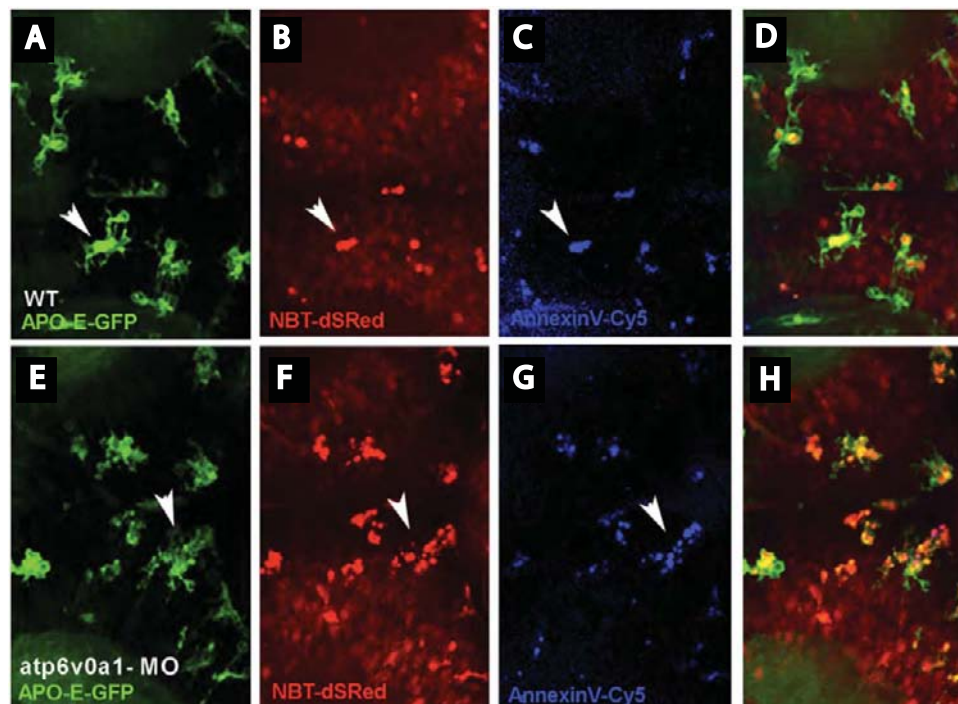


Plate 47. Images of microglia consuming dying neurons by phagocytosis in wild-type and *atp6v0a1* morphant larvae. See [Figure 36-6](#) for details.

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1. INTRODUCTION

The gastrointestinal (GI) tract begins with the mouth, leads to the esophagus, and extends through the stomach, small intestine (including duodenum, jejunum, and ileum), and large intestine (divided into cecum and colon), to end at the anus. In addition, the GI tract includes three accessory organs: liver, gallbladder, and pancreas. The liver produces bile, a fluid containing molecules (bile acids) that help the digestion of lipids, and, via numerous canaliculi forming the biliary system, secretes it into the gallbladder, where it is stored and concentrated. Upon eating, bile is discharged into the small intestine. The pancreas is a dual-function gland, working as both an endocrine and an exocrine gland. The exocrine pancreas secretes pancreatic juice containing bicarbonate and several enzymes, including trypsin, chymotrypsin, lipase, and pancreatic amylase, into the small intestine. Both the liver and the pancreas aid in the digestive process.

The gastrointestinal epithelium is characterized by rapid proliferation of stem cells that differentiate to become mature cells. At the same rate, older and/or damaged cells are eliminated by apoptosis, and the resulting apoptotic bodies are shed into the lumen and/or engulfed by adjacent epithelial cells and subepithelial macrophages. This highly regulated balance between cell proliferation and apoptosis ensures the maintenance of tissue function and architecture. Conversely, alterations in the rate of cell proliferation or cell death result in the development of pathologic states. Indeed, apoptosis has been shown to play an important role in the pathophysiology of several gastrointestinal diseases. Many infectious and immune-mediated diseases, such as gastritis, viral hepatitis, and inflammatory bowel diseases, may be triggered by excessive cell

death, whereas prolonged cell survival due to apoptosis inhibition, together with unregulated proliferation, can promote cancer development. This chapter reviews the current knowledge of the role and mechanisms of apoptosis in the organs of the GI tract under physiologic and pathological conditions.

2. ESOPHAGUS

The esophageal epithelium is a nonkeratinized, stratified squamous epithelium, with scattered submucosal glands that produce mucus and provide lubrication. The esophageal epithelial cells normally undergo a rapid turnover to eliminate and replace cells mechanically and chemically damaged during the transit of food. New cells are generated by the division of stem cells located in the basal compartment of the squamous epithelium; at each cell division, these cells give rise to one stem cell (to maintain the stem cell pool) and one daughter cell, which differentiates into mature epithelial cell and eventually undergoes apoptosis after a number of divisions, ensuring a functional tissue homeostasis. However, when the balance between cell proliferation and cell death is lost, the epithelial integrity and architecture are altered, with serious pathological consequences. A common example is represented by a condition known as Barrett's esophagus (BE), during which the squamous epithelium is transformed into a metaplastic simple columnar epithelium, which resembles that of gastric mucosa or of intestinal mucosa (*vide infra*). BE is considered a premalignant condition and is associated with an increased risk for the development of esophageal adenocarcinoma (ADCA). This pathology is often the consequence of chronic inflammation caused by gastroesophageal reflux disease (GERD), a condition characterized by backflow of the gastric contents into the

esophagus. The two major components of the reflux responsible for the development of BE are gastric acid and bile acids. Bile acids cause apoptosis and, in an acidic environment, they are able to rapidly induce oxidative stress and oxidative DNA damage. Therefore, esophageal cells exposed to the refluxate are initially subjected to a faster turnover, mainly controlled by the tumor suppressor genes p16 (also known as CDKN2A or p16[INK4]) and p53, which regulate cell cycle arrest and DNA damage-induced apoptosis, respectively. A persistent exposure ultimately increases the risk of genetic instability, resulting in clonal selection of a cell population bearing alterations in gene expression that promote increased cell division, apoptosis resistance, invasion, and metastasis. Indeed, normal squamous epithelium is sensitive to bile acid-induced apoptosis, whereas BE metaplastic cells become resistant. Consistently, inactivating mutations of p16 and p53 genes through promoter methylation, gene mutation, or loss of heterozygosity are common early events in the progression from BE to ADCA. Other factors contributing to increased apoptosis-resistance of metaplastic cells include over-expression of the antiapoptotic proteins Bcl-X_L and Mcl-1, interleukin (IL)-6, and cyclo-oxygenase-2 (COX2); decreased cell-surface expression of Fas (CD95) and increased Fas ligand (CD95L) expression. In particular, acid exposure has been shown to increase COX2 expression through activation of both extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways, which cause a significant increase in COX2 promoter activity. COX2 expression is also induced by the activation of nuclear factor kappa B (NF- κ B), a transcription factor constitutively activated in chronic inflammatory conditions. Moreover, specific bile acids may also directly activate the PI3 kinase/Akt signaling pathway, which stimulates cell growth and inhibits apoptosis in Barrett's adenocarcinoma cells, therefore promoting neoplastic progression of BE. Thus dysregulation of apoptosis plays a central role in the progression to a malignant phenotype.

3. STOMACH

The gastric epithelium is made up of a single layer of cells indented into numerous short gastric pits. The epithelium consists of only one cell type, the surface mucous cells, which secrete mucus to protect the stomach surface from digestive acid and enzymes. Beneath the gastric pits, the mucosa is filled with long contiguous tubular glands divisible into isthmus, neck, and base regions. The gastric glands consist primarily of

two cell types: (1) the acid-secreting parietal (oxyntic) cells, found mainly in the neck region; and (2) the pepsinogen-secreting chief cells, usually located in the base region. The glands also contain mucous neck cells (in the neck area) and stem cells, located at the top of the glands (isthmus), where they open into the pits. The stem cells (or progenitor cells) undergo frequent mitosis to propagate themselves and to generate new gland cells and surface mucous cells. The newly generated cells migrate either outward into the pit, mature into surface mucous cells, and proceed toward the surface where they are eventually eliminated, or inward to the neck region where they differentiate into mucous neck cells, parietal cells, and chief cells. The turnover of surface epithelial cells is fairly rapid, with the entire epithelium replaced within 3 to 5 days, whereas parietal and chief cells die at a lower frequency. Under normal conditions, surface mucous cells constantly undergo apoptosis, and this rapid self-renewal of the epithelium serves as a host defense mechanism to limit bacterial colonization. However, some bacteria have developed the capacity to evade the defense mechanisms by interacting with the host epithelium. The most remarkable example is *Helicobacter pylori*, a Gram-negative spiral bacterium that chronically infects up to 50% of the human population, the infection of which has been associated with severe gastric pathologies, including gastritis and peptic ulcer. Chronic *H. pylori* infection is also the strongest known risk factor for the development of gastric cancer. This microorganism is able to invade and colonize human stomach by directly interacting with gastric epithelial cells, resulting in alterations of cell cycle and apoptosis in the host cell. *H. pylori* inhibits apoptosis in the directly infected gastric epithelial cells to facilitate its persistence within the human stomach, contributing to pit hyperplasia and persistent infection of the stomach. At the same time, the chronic infection and subsequent inflammatory response lead to loss of uninfected parietal cells and chief cells, resulting in oxyntic atrophy and gastric metaplasia, both pathological conditions predisposing to gastric cancer. *H. pylori* infection results in activation of ERK- and NF- κ B-mediated prosurvival signaling pathways, leading to growth factor upregulation (in particular gastrin and COX2), gastric epithelial proliferation, cell-cell dissociation and increased cell motility, and over-expression of the antiapoptotic protein Mcl-1 in the gastric pits. These effects are mediated by the cytotoxin cytotoxin-associated antigen A (CagA), which is injected by the bacterium into the gastric epithelial cell. Consistently, infections with CagA-positive strains of *H. pylori* are associated with

the highest risk of developing gastric cancer. Chronic *H. pylori* infection also triggers a persistent immune response, resulting in chronic inflammation with production of inflammatory cytokines (especially IL-6 family cytokines) and oxygen-free radicals, the latter produced by polymorphonuclear cells and macrophages infiltrating the gastric mucosa. The inflammatory milieu favors the onset of genetic mutations via direct DNA damage and impaired DNA repair and predisposes to neoplastic transformation through inhibition of apoptosis, resistance to immune response, and stimulation of angiogenesis.

4. SMALL AND LARGE INTESTINE

The simple columnar epithelium of the intestine is made up of highly specialized cells (enterocytes or intestinal epithelial cells [IECs]) whose primary function is to absorb and transport nutrients across the epithelial lining while maintaining a physical barrier to the external environment. Scattered among the enterocytes are other cell types, including goblet cells (specialized in secretion of mucus to facilitate passage of material through the bowel), Paneth cells (similar to neutrophils and providing host defense against microbes), enteroendocrine cells, and occasional infiltrating lymphocytes and eosinophils. To help maintain a barrier, the epithelial cells are joined by tight junctions on their lateral borders, thus limiting the passage of luminal contents across the cell. Within the small intestine, efficient absorption is facilitated through finger-like projections called villi, which greatly enhance the surface area. Unlike the small intestine, the large intestine does not contain villi. Throughout the intestine, flask-like structures called crypts contain rapidly proliferative cells responsible for maintaining epithelial integrity through constant production of new cells. In the small intestine, the crypts are located around the base of the villi, and new cells generated from the stem cells located at the bottom of the crypt move up the crypt–villus axis while undergoing the differentiation process. In the colon, the new cells migrate from the crypts toward the table region. Under normal conditions, spontaneous apoptosis is observed at two locations: (1) at the base of the small intestinal crypts, where it is believed to occur to control the stem cell population by removing excess and/or damaged cells; and (2) at the top of the villi (in the small intestine) or toward the top of the colonic crypt (in the large intestine), where aging and/or damaged epithelial cells are eliminated mainly by an apoptotic process triggered by the loss of cell–matrix interactions

during the progressive detachment of the cell, a form of cell death referred to as anoikis.

The expression of several members of the Bcl-2 family has been studied throughout the normal intestinal tissue and has been found to correlate with the levels of spontaneous apoptosis. For example, the antiapoptotic protein Bcl-2 is strongly expressed at the base of the colonic crypts, where virtually no spontaneous apoptosis of stem cells occurs, whereas it is absent in the crypts of the small intestine, where levels of spontaneous apoptosis are significantly higher. Conversely, the proapoptotic proteins Bax and Bak are highly expressed in the crypts of the small intestine, but weakly expressed within the colonic crypts. The distribution of these pro- and antiapoptotic Bcl-2 proteins may explain, at least in part, the increased risk of developing cancer in the large intestine as compared with the small intestine. Spontaneous apoptosis is, indeed, more frequent in the small intestine and provides a tight regulation of stem-cell homeostasis, preventing the generation of hyperplastic crypts with higher disposition to neoplastic transformation.

To avoid compromising the epithelial integrity, the enterocytes have developed mechanisms to sustain the epithelial barrier function during spontaneous apoptosis. However, excessive apoptosis can lead to depletion of crypt stem cells, shortening of the crypt–villus axis due to inability to compensate the cell loss at the villus tip, and ultimately, epithelium destruction and intestinal atrophy, which is associated with several gastrointestinal diseases. This represents a significant therapeutic problem for the use of abdominal and pelvic radiotherapy and chemotherapy-based treatments of cancer patients, as these treatments are known to induce severe intestinal damage as a result of intestinal stem-cell apoptosis. Although the precise mechanisms that regulate repair and survival of the intestinal crypt are still elusive, a recent study established a critical role for the BH3-only p53-upregulated modulator of apoptosis (PUMA) protein in radiation-induced apoptosis of intestinal progenitor and stem cells and subsequent intestinal damage (Figure 21-1). Moreover, high concentrations of cytokines such as tumor necrosis factor- α (TNF- α) and interferon- γ , as found in an inflammatory milieu, can directly induce epithelial apoptosis and disrupt tight junction formation, thereby weakening barrier function. Indeed, dysregulated apoptosis and changes in enterocytic junctions have been involved in the pathogenesis of inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis. In patients with IBD, increased apoptosis is found in the acute inflammatory sites throughout the entire

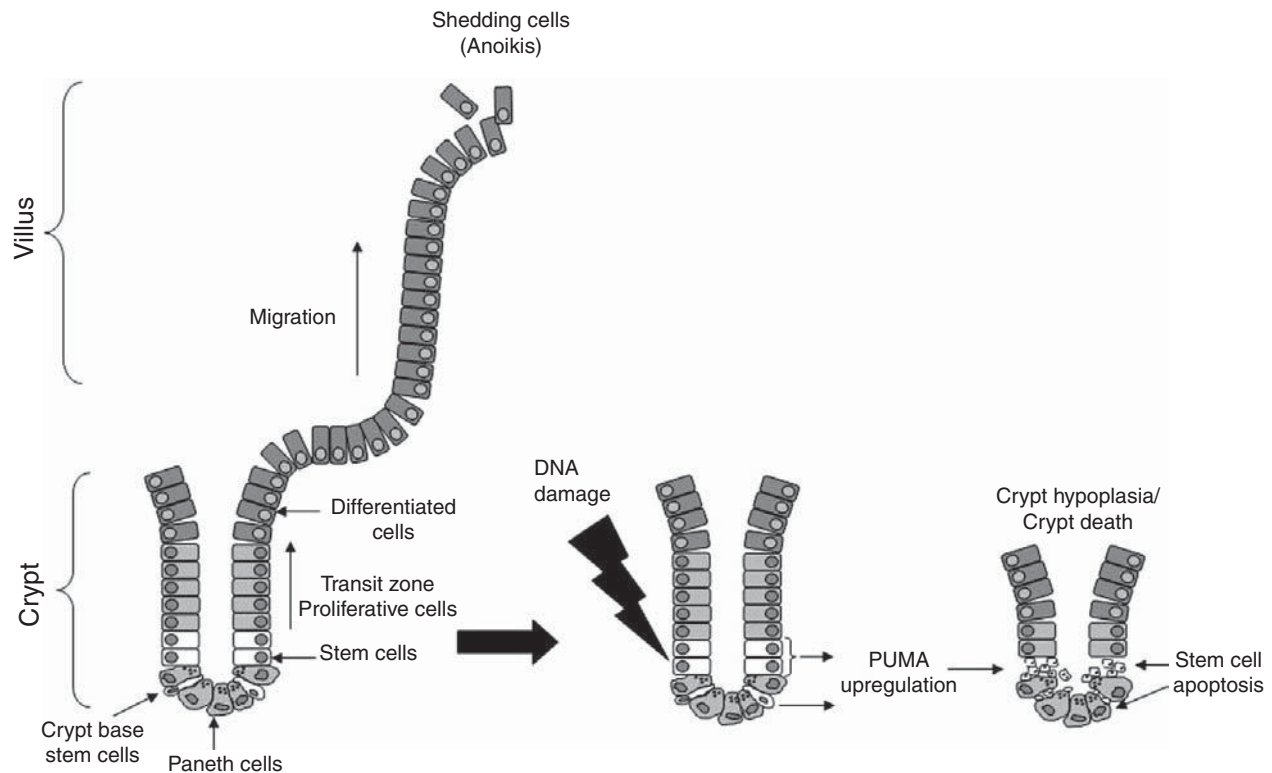


Figure 21-1. Schematic representation of small intestinal epithelium showing cell proliferation and death in crypts and villi under normal and pathological conditions. Stem cells located in the basal region and just above the crypt base divide asymmetrically to generate one stem cell and one daughter cell, which undergoes further division and differentiation as it migrates up toward the tip of the villus, where cells are eventually shed by anoikis. When DNA damage occurs (i.e., ionizing radiations, chemotherapy), the p53 target protein PUMA is upregulated in the stem cells, leading to increased stem-cell apoptosis and crypt loss.

crypt–villus axis. This increased epithelial apoptosis is caused by chronically activated lamina propria T lymphocytes of the intestinal mucosa, which can directly kill the intestinal epithelial cells mainly via the Fas/FasL pathway and also produce high levels of proinflammatory cytokines such as TNF- α , IL-6, and interferon- γ , resulting in chronic mucosal inflammation and colonic tissue damage. Moreover, recent studies demonstrated that constant interfacing with microbes in the gut lumen results in endoplasmic reticulum (ER) stress and triggers a consequent unfolded protein response in the intestinal epithelial cells to restore ER homeostasis. Mutations in one key mediator of this ER stress response, X-box-binding protein 1 (XBP1), have been associated with increased apoptosis and development of IBD, suggesting that ER stress-mediated apoptosis plays a crucial role in the pathogenesis of IBD and that intestinal epithelial cells may perform homeostatic functions in the gut in addition to the immune cells (Figure 21-2). Persistent intestinal epithelial cell apoptosis eventually leads to disruption of the epithelial barrier function, facilitating the invasion of pathogenic microorganisms.

Conversely, an imbalance between cell proliferation and apoptosis in favor of proliferation predispose to the development of colorectal carcinoma. This cancer progresses through a multistep transformation of normal colonic epithelium to an adenomatous polyp and, ultimately, to invasive carcinoma, characterized by an accumulation of genetic alterations leading to an increasingly malignant phenotype. These mutations generally affect genes regulating cell proliferation and apoptosis in cells that ultimately acquire resistance to cell death and accumulate at the top of the crypt and surface epithelium, contributing to neoplastic transformation. Indeed, spontaneous apoptosis is progressively decreased as the colonic cell progresses from normal epithelium to sporadic adenoma to carcinoma. One of the most commonly mutated genes involved in regulation of cell cycle and apoptosis is *p53*, which is absent or mutated in 75% to 85% of all human colon cancers. Mutations in the *p53* gene occur late in the adenoma-to-carcinoma sequence of colon cancer progression and may allow the growing tumor with multiple genetic alterations to evade cell cycle arrest and apoptosis. Induction of wild-type

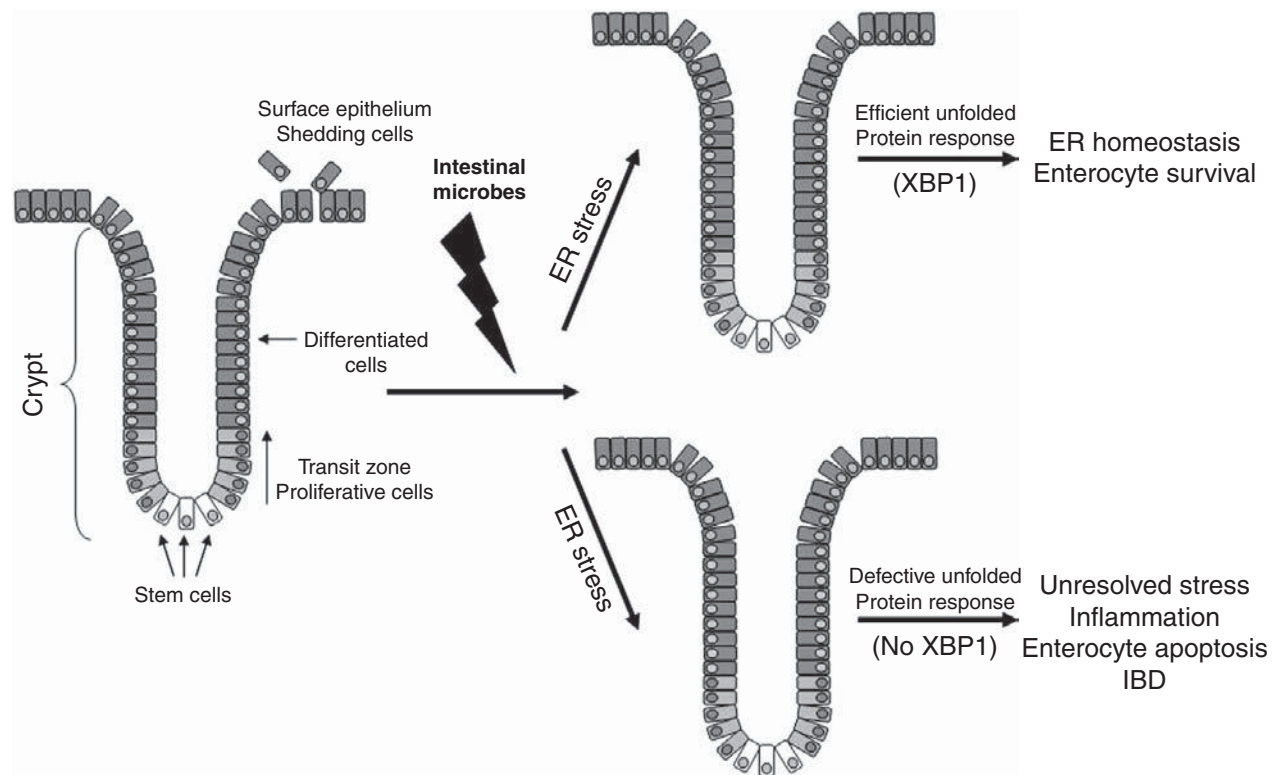


Figure 21-2. Schematic representation of the colonic crypt showing cell proliferation and death under normal and pathological conditions. Stem cells located at the bottom of the crypt divide asymmetrically to generate cells that undergo further division and differentiation as they move toward the top of the crypt (see Figure 21-1). Molecules produced by gut microbes trigger constant ER stress in the intestinal epithelial cells, which activates the unfolded protein response to maintain ER homeostasis. Defective unfolded protein response (i.e., lack of functional XBP1) leads to enterocyte apoptosis and promotes development of inflammatory bowel disease.

p53 results in both cell cycle arrest by transcriptional upregulation of the cyclin kinase-dependent cell cycle inhibitor $p21^{\text{Waf1/Cip1}}$ and apoptosis by upregulation of proapoptotic genes and direct induction of mitochondrial permeabilization via activation of Bax. Another genetic change often observed in colorectal carcinoma is over-expression of Bcl-2, which is no longer restricted to the crypt base, but it becomes detectable throughout the entire malignant epithelium. Bcl-2 expression increases only in the early stages of the progression from adenoma to carcinoma, contributing to the inhibition of apoptosis during the initial stages of tumorigenesis, and decreases thereafter. However, apoptosis remains impaired even in the presence of reduced Bcl-2 due to the onset of other antiapoptotic changes, including *p53* mutations and over-expression of Bcl-X_L. Over-expression of cellular FLIP (cFLIP), a potent inhibitor of death receptor-mediated apoptosis, is also a frequent event in the development of colon carcinoma, contributing to the progression from adenoma to carcinoma and increasing resistance to chemotherapy-induced apoptosis. In addition to inhibiting apoptosis, cFLIP has also

been shown to directly activate ERK-mediated survival pathways and to promote tumorigenesis by activating the Wnt/ β -catenin pathway. Other genes involved in the regulation of cell proliferation and/or apoptosis are also commonly mutated in colorectal carcinomas. Among those, inactivating mutations of both alleles of the adenomatous polyposis coli (APC) gene, a tumor suppressor involved in regulation of the adherens junction protein β -catenin, are a frequent early event in the development of sporadic colorectal cancers. Mutations in the *K-ras* proto-oncogene also occur early in the adenoma stage and can increase proliferation and inhibit apoptosis. Finally, death receptor-mediated apoptosis, in particular Fas- and TNF-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis, is crucial to eliminate cells bearing genetic alterations by the immune cells. However, colon cancer cells have been found to express Fas ligand early in the adenoma-to-carcinoma sequence, which allows them to eliminate Fas-expressing lymphocytes and create sites of immune privilege. Moreover, despite the expression of Fas, most colon cancer cells are resistant to Fas-mediated apoptosis due

to the previously mentioned genetic alterations in the apoptotic machinery, which can include p53 mutations, upregulation of Bcl-2 and cFLIP, and downregulation of Bax. The same mutations may also account for resistance to TRAIL-mediated apoptosis acquired by some colon carcinoma cell lines, despite even higher levels of TRAIL receptors in tumor cells as compared with normal colonic epithelium.

5. LIVER

The liver is a complex and exceptionally specialized organ with metabolic, synthetic, and detoxifying functions. This complexity is reflected in its cellular composition, which includes several cell populations: (1) hepatocytes, multifunctional epithelial cells representing the vast majority of liver parenchyma; (2) hepatic stellate cells (HSC), located in the space of Disse and involved, among other functions, in the fibrogenic process; (3) sinusoidal endothelial cells, which form a fenestrated endothelium that lines the liver sinusoids and allow direct contact between hepatocytes and plasma; (4) Kupffer cells (KCs), liver macrophages located within the lumen of the liver sinusoids and involved in the liver's response to toxins, infections, and other stresses; (5) biliary epithelial cells or cholangiocytes, which line the lumen of the biliary tree and participate in bile formation. For its unique nature and anatomical location, the liver is exposed to a multitude of toxins and infectious agents coming from the gastrointestinal tract through the portal blood flow and therefore is highly susceptible to tissue injury. To protect itself, the liver has evolutionarily developed several adaptive responses, including an exquisite sensitivity to apoptosis to promptly eliminate damaged or infected cells and a unique ability to regenerate up to 70% of its tissue to counterbalance the cell loss. However, when the cellular loss exceeds the liver regenerative capacity, the organ is no longer able to fulfill its functions, and hepatic failure occurs. In many acute and chronic liver diseases, such as viral and autoimmune hepatitis and cholestatic disease; after chronic exposure to toxins, drug, or alcohol; or in transplantation-associated liver damage, including ischemia-reperfusion injury and graft rejection, regeneration may not fully compensate the tissue loss caused by excessive apoptosis. Moreover, engulfment of greater amounts of apoptotic bodies by KCs may, in these conditions, exacerbate liver inflammation and tissue damage by amplifying death receptor-mediated hepatocyte apoptosis through production of FasL and TNF- α by the KCs themselves. As a result, functional hepatic parenchyma is gradually replaced by fibrotic scar tissue synthesized largely

by activated HSCs, eventually preventing the liver from functioning properly, a condition referred to as liver fibrosis or, in its end-stage, liver cirrhosis. The scar tissue blocks the flow of blood through the organ and drastically slows its functions until hepatic failure occurs. Therefore, excessive hepatocyte apoptosis promotes the development of hepatic fibrosis. Conversely, as activated HSCs are the main source of type I collagen, the principal matrix protein responsible for the development of fibrosis, a therapeutic approach aimed to selectively eliminate them could potentially be beneficial to attenuate liver fibrosis.

Several stimuli can induce apoptosis in the liver, but its cells are particularly sensitive to death receptor-mediated apoptosis as a result of the relatively high level of expression of the four most important death receptors, Fas, TNF-receptor 1 (TNF-R1), TRAIL receptor 1, and TRAIL receptor 2. This enhanced death receptor expression is likely the result of evolutionary pressure to support the vital need for the liver to eliminate virus-infected and/or mutated cells, which is achieved via engagement of death receptor-mediated apoptotic pathways by death ligand-expressing immune cells. Indeed, both TRAIL- and Fas-mediated apoptosis are essential components of cancer immunosurveillance by T cells and natural killer cells in the liver. However, this high sensitivity to death receptor-mediated cytotoxic pathways can also expose the liver to massive tissue damage if the receptors are excessively or chronically activated. For example, liver damage during viral hepatitis, a disease generally caused by infection with hepatitis B or hepatitis C virus, largely results from death receptor-mediated apoptosis associated with the host immune response to the viral antigen and not from a direct cytotoxic effect of the virus. Similarly, death receptor-mediated apoptosis has been involved in the pathogenesis of several other liver diseases, including alcoholic hepatitis, cholestatic liver disease, Wilson's disease, and nonalcoholic steatohepatitis (NASH). For example, the liver of patients with NASH shows elevated Fas expression and increased sensitivity to Fas-mediated apoptosis. Moreover, hepatocyte growth factor (HGF) receptor, Met, usually associates directly with Fas in normal liver, thus preventing its activation by FasL. Recent studies showed that in steatotic livers, this association is disrupted, whereas HGF and FasL expression is elevated, resulting in increased hepatocyte apoptosis and liver injury. The same studies also demonstrated that the use of a synthetic peptide mimicking the Fas-binding motif on Met effectively protects the liver from Fas-mediated apoptosis and liver damage, demonstrating that Fas apoptosis is a key event in the pathogenesis

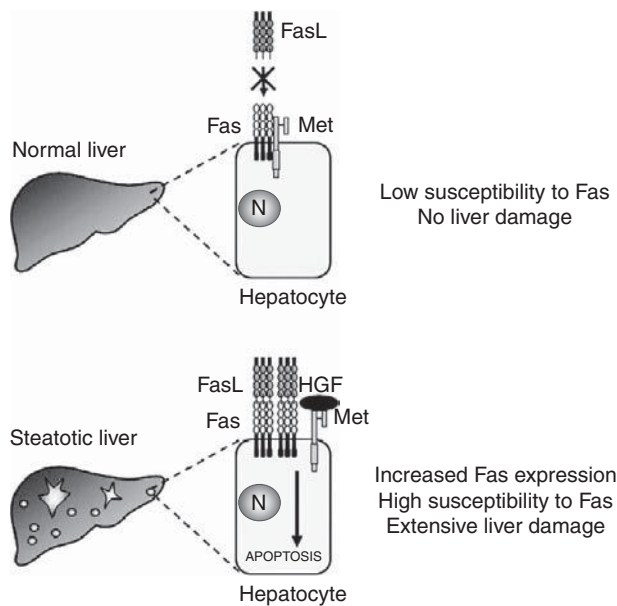


Figure 21-3. Schematic representation of different susceptibility of hepatocytes to Fas-mediated apoptosis in normal and steatotic livers. In normal hepatocytes, the HGF receptor Met associates with Fas on the plasma membrane, preventing its activation by FasL. In hepatocytes from steatotic livers, Fas is over-expressed and no longer associates with Met; in addition, FasL and HGF are also over-expressed, resulting in increased hepatocyte apoptosis and liver injury.

of NASH (Figure 21-3). Death receptor engagement, however, does not always result in cell death. In particular, nonapoptotic signals can be activated by death ligands in cells that have acquired resistance to apoptosis through mutations of crucial components of the apoptotic machinery, including, but not limited to, loss-of-function mutations in the death receptor gene, upregulation of antiapoptotic Bcl-2 family members or cFLIP, or downregulation of proapoptotic Bcl-2 proteins. In these conditions, death receptors have been shown to promote oncogenic features, such as tumor proliferation, metastasis, and invasion via activation of NF- κ B- and MAPK-regulated survival pathways. Therefore, an insult to the liver (i.e., viral infection, alcohol intake) generates an initial apoptotic response mediated by the immune cells that aims to eliminate the damaged or infected cells. If the exposure to the toxic agent persists, excessive apoptosis and chronic inflammation may favor the accumulation of genetic mutations, in particular mutations of tumor suppressor genes (i.e., *p53*) and genes involved in apoptosis signaling, and promote development of hepatocellular carcinoma. If the cancer cells have acquired resistance to death receptor-mediated apoptosis, engagement of death receptors could actually result in generation of a more aggressive phenotype. This observation has great clinical relevance in particular for

TRAIL, the use of which in cancer therapy is currently under evaluation.

6. PANCREAS

The pancreas is divided into lobules by septae of connective tissue. Each lobule is composed largely of grape-like cluster of exocrine cells called acini, which secrete the pancreatic juice containing digestive enzymes. This is collected into a tree-like series of ducts that run within the organ and is finally delivered into the duodenum through the main pancreatic duct. Scattered within the pancreatic exocrine tissue are clusters of cells called islets of Langerhans, which represent the endocrine component of the pancreas and produce several important hormones, including insulin, glucagon, and somatostatin. For the purpose of this chapter, we focus only on the exocrine component of the pancreas as a functional part of the GI system.

Apoptosis is involved in both development and progression of several pancreatic diseases, including acute and chronic pancreatitis and pancreatic ductal adenocarcinoma (PDAC). Acute pancreatitis is a disease associated with variable severity from mild, self-limited attacks, to severe, highly morbid, and frequently lethal attacks. The first acinar cell response to the injury seems to be the main factor determining the disease severity. In particular, extensive apoptosis of acinar cells is associated with mild acute pancreatitis, whereas severe acute pancreatitis involves extensive acinar cell necrosis, but very little acinar cell apoptosis. Little is known about the mechanism of apoptosis in the pancreatic acinar cells, but evidence of mitochondrial dysfunction points to the involvement of the intrinsic pathway of apoptosis. The massive release of cellular content occurring during necrotic cell death is likely responsible for recruitment of inflammatory cells and generation of inflammatory mediators, which negatively affect the course of the disease. NF- κ B, which plays a critical role in the inflammatory response by regulating transcription of inflammatory cytokines such as TNF- α , IL-1 β , and IL-6, is activated early in pancreatic acinar cells in models of experimental acute pancreatitis. Among the first response to NF- κ B activation in acinar cells is the induction of the acute phase protein pancreatitis-associated protein-1 (PAP1), which reduces the extent of necrosis and infiltration of immune cells. Recent studies have shown that functional inactivation of NF- κ B in acinar cells increases the susceptibility of these cells to inflammation-induced cell death and results in severe necrotizing pancreatitis, supporting a protective and organ-specific function of NF- κ B during acute pancreatitis.

Chronic pancreatitis is characterized by progressive loss of acinar parenchyma and aggressive fibroinflammatory reactions, ultimately leading to irreversible organ destruction. Chronic inflammation induces neo-expression of death receptors (especially Fas and TRAIL receptors) in pancreatic acini, which become susceptible to apoptosis triggered by death ligands expressed on lymphocyte and released by pancreatic stellate cells (PSCs). In contrast, islets remain relatively intact because of failure to express functional death receptors and activation of NF- κ B-induced antiapoptotic factors. Activation of PSCs plays a crucial role in pancreatic fibrogenesis during chronic pancreatitis; several inflammatory mediators, including transforming growth factor-beta (TGF- β), platelet-derived growth factor (PDGF), IL-1, IL-6, and TNF- α , stimulate the phenotypic changes from quiescent to active form.

Chronic inflammation is also a well-recognized risk factor for the development of PDAC. In PDAC, a variety of growth factors and growth factor receptors are expressed at increased levels. For example, over-expression of both EGF receptor and its ligands EGF and TGF- α is often observed in PDAC and is associated with enhanced tumor aggressiveness and shorter survival after tumor resection. In addition, PDAC often exhibits alterations in growth inhibitory pathways, such as Smad4 mutations and Smad6 and Smad7 over-expression, and evades apoptosis through mutations of tumor suppressor genes such as *p53* and *p16* and aberrant expression of apoptosis-regulating genes, including members of the Bcl-2 family. These alterations combined give pancreatic cancer a distinct growth advantage that clinically results in rapid tumor progression and poor survival prognosis. Also, pancreatic carcinoma cells have developed different mechanisms to evade the host immune surveillance. One is the expression of nonfunctional receptors (decoy receptors) and antiapoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-X_L. Another is the expression of apoptosis-inducing ligands, such as FasL and TRAIL, which are able to trigger apoptosis of immune cells, creating areas of immune privilege. Similarly to what is observed for hepatocarcinomas, successful treatment of malignant tumors by recombinant TRAIL might be possible in some cases, but not in all pancreatic tumors, because of their differential resistance to TRAIL-induced cell death.

7. SUMMARY AND CONCLUDING REMARKS

The GI tract is characterized by a very dynamic epithelium that undergoes constant renewal to ensure

replacement of cells that have been damaged by toxins and/or microorganisms entering the body with food. Apoptosis plays a fundamental role in this process by counterbalancing the number of cells generated by division and differentiation of precursor stem cells. Over-activation of apoptosis can lead to significant tissue damage, whereas inhibition of apoptosis can promote proliferation and oncogenic transformation of cells. Cells bearing chromosomal alterations or mutated DNA are generally eliminated by apoptosis, mainly via activation of p53, thus preventing malignant transformation. Indeed, immortalization, a process characterized by unlimited potential to divide and resistance to cell death, is an important event in tumorigenesis, which probably occurs early in the neoplastic process. Consistently, many infectious and immune-mediated GI diseases, such as gastritis, viral hepatitis, and IBD, are initially associated with increased apoptosis and tissue damage, which generates inflammatory, fibrogenetic, and immune reactions accompanied by alterations of cellular growth and death, often resulting in cancer of the organ. Chronic inflammation, in particular, is a known predisposing factor to the development of cancer in the GI tract. Thus therapeutic approaches aiming to modulate apoptosis have the potential to be an effective tool for treating GI diseases.

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22

Apoptosis in the Kidney

Juan Antonio Moreno, Adrian Mario Ramos, and Alberto Ortiz

1. NORMAL KIDNEY STRUCTURE AND FUNCTION

The kidneys maintain the homeostasis of electrolyte, fluid, and acid–base balance; eliminate waste products; and have an endocrine-metabolic function. They secrete hormones such as erythropoietin, Klotho, and 1,25-(OH)₂-vitamin D and clear other hormones and cytokines. Each kidney contains 1 million basic functional units, or nephrons. Each nephron is composed of a glomerulus and a renal tubule. The glomerulus is a tightly woven, highly permeable capillary bed, surrounded by differentiated, very specialized cells, the podocytes. The mesangium contains mesangial cells and holds the capillaries together. Every day, 180 L of plasma is filtered through the glomeruli. Podocytes prevent the filtration of proteins, and their injury will lead to pathological urinary protein excretion (proteinuria). Podocytes do not divide, and podocyte loss causes podocytopenia, an early event in progressive glomerular scarring. Tubular cells reabsorb most of the filtered fluid and nutrients, and only 1 to 2 L of urine is excreted. Proximal tubular cells are responsible for the bulk of reabsorption. They are rich in mitochondria, consume high amounts of energy, and express a variety of transporters that favor the uptake of nephrotoxins. Thus they are prime targets in toxic and ischemic renal injury.

2. APOPTOSIS IN KIDNEY DEVELOPMENT AND CONGENITAL KIDNEY DISEASES

Normal nephrogenesis results from finely balanced proliferative and apoptotic cell death processes. The ureteric bud invades the metanephric mesenchyme, branching and promoting the differentiation of the mesenchyme

into nephrons. The metanephric mesenchyme has a default fate of apoptosis that is prevented by factors secreted from the bud, such as transforming growth factor (TGF)- α , epidermal growth factor, fibroblast growth factor 2, and glial cell line–derived neurotrophic factor (GDNF). Genetic evidence from knockout mice indicates that during development, the high expression of Bcl-2 and Pax-2 protects cells against apoptosis, allowing cell proliferation and differentiation. In the mature kidney, Pax-2 is not found, and the expression of Bcl2 is low. Other antiapoptotic molecules, such as Bcl-xl, predominate. However, in the course of renal injury, adult kidneys may re-express some of these antiapoptotic factors in the frame of a more general adaptive response against the aggression.

Bcl-2–deficient mice (*bcl-2*^{−/−}) are viable. However, they die within a few months of birth from renal failure. Renal hypoplasia and cystic dysplasia resembling polycystic kidney disease (PKD) result from excessive apoptosis in the metanephric blastema and nephrogenic zones. Bim is a key factor in renal injury in *bcl-2*^{−/−} mice. Normal kidney development is restored in *bcl-2*^{−/−} *bim*^{+/−} chimeric mice. Bim is not required for normal renal development because kidneys from *bim*^{−/−} mice are normal. This has been explained by the existence of a hierarchical functional axis involving Bim, Bcl-2, and Bak/Bax. Active Bim might initiate the death signaling acting as a sensor setting the apoptotic threshold, whereas Bcl-2 might or might not allow the propagation of death stimulus according to its expression level. Bak/Bax might execute the death program depending on the result of the Bim and Bcl-2 interaction.

Apoptotic loss of cells is a hallmark of renal hypoplasia, a developmental disease with a genetic

Table 22-1. Main renal diseases with renal cell loss by apoptosis

Renal disease	Main cell type undergoing apoptosis	Main apoptosis inducers
Renal hypoplasia/agenesis	Metanephric mesenchyme and ureteric bud cells	Absence of survival factors
Acute kidney injury	Tubular cells	Nephrotoxins, ischemia/reperfusion, inflammatory mediators
Chronic kidney disease	Podocytes, glomerular mesangial and endothelial cells, tubular cells	Inflammatory mediators, etiology-specific factors
Diabetic nephropathy ^a	Podocytes, tubular cells	High glucose, glucose degradation products, extracellular matrix alterations, inflammatory mediators (angiotensin II; TGF β 1, TNF superfamily cytokines)
Vascular renal injury ^a	Tubular cells	Ischemia
Glomerular injury ^a	Podocytes, mesangial cells	Inflammatory mediators
Polycystic kidney diseases ^a	Tubular cells	Genes encoding ciliary proteins

^a Diabetic nephropathy, vascular renal injury, glomerular injury, and polycystic kidney diseases are the most frequent causes of CKD.

basis. Homozygous or heterozygous mutations of the antiapoptotic Pax-2 gene result in a variable pathology ranging from bilateral renal agenesis and severe renal hypoplasia to mild renal hypoplasia. Low *pax-2* expression does lead to changes in Bcl-2 expression. However, targeted over-expression of Bcl2 reverses the programmed cell death observed in the ureteric bud of *pax2*^{-/-} mutant mice and restores normal kidney size, nephron number, and renal function. Heterozygous mutations in RET, the GDNF receptor, may result in renal agenesis in humans.

3. APOPTOSIS IN ADULT KIDNEY DISEASE

Disturbances in cell number in which apoptosis is involved have been described in animal models and clinical renal diseases. We review the role and regulation of apoptosis in acute kidney injury (AKI), chronic kidney disease (CKD), diabetic nephropathy, glomerular injury, and PKD. An imbalance between mitosis/chemotaxis and apoptosis can result in disorders of cell number characterized by an excessive cell number (e.g., proliferative glomerulonephritis) or insufficient cell number (e.g., renal atrophy) (Table 22-1). Although dysregulated fibroblast or leukocyte apoptosis may contribute to renal fibrosis and inflammation, respectively, we concentrate here on parenchymal renal cell apoptosis. Loss of parenchymal renal cells characterizes both AKI and CKD. Podocytes and tubular cells

may be lost by shedding, death, or differentiation into fibroblasts. All of these mechanisms are responses to injury that may coexist and contribute to renal cell loss. To date there is insufficient information on the relative contribution of each of them to cell loss in many disease processes. Apoptosis may be the initial insult that causes renal disease, or it may contribute to progressive renal cell loss. However, apoptosis is also required for tissue remodeling and recovery of normal tissue structure. As an example, redundant cells in proliferative glomerulonephritis are cleared through apoptosis. Thus it is important to understand the kinetics, targets, and mechanisms of apoptosis in preclinical models before planning clinical trials of antiapoptotic drugs in kidney disease (Table 22-2).

AKI is a syndrome characterized by an acute loss of renal function. Because of its time frame, it is the model of kidney injury in which the role and regulation of apoptosis has been most extensively studied. Current therapy of AKI is symptomatic and consists of substitution of renal function by dialysis if renal failure is severe. There is no established therapy to accelerate the recovery, and attempts at preventing AKI are not universally effective. Despite the reversibility of the loss of renal function, the mortality of AKI remains high (>50%). Thus therapies based in a correct understanding of its pathogenesis are urgently needed. In human studies, tubular cell death is the best histological correlate with renal dysfunction in AKI. Evidence supporting a key role of

Table 22-2. Role of apoptosis in kidney disease

Cell target	Timing	Problem	Consequence
Podocytes	Acute, chronic	Cell death in nondividing cells	Podocytopenia, glomerulosclerosis, CKD progression
Tubular cells	Acute, chronic	Cell death exceeds mitotic potential	AKI, tubular atrophy, CKD progression
Mesangial cells	Chronic	Cell death exceeds mitotic potential	Glomerulosclerosis, CKD progression
Mesangial, tubular cells	Recovery phase (reactive hyperplasia)	Cell death exceeds mitotic rate transiently to eliminate excessive cells	Restoration of normal cell number
Inflammatory cells	Acute, chronic	Insufficient apoptotic clearance	Persistent inflammation
Fibroblasts	Chronic	Insufficient apoptotic clearance	Failure to resolve fibrosis, progressive fibrosis, CKD progression

Note: Apoptosis may be beneficial or deleterious in the course of kidney disease, depending on the magnitude of the phenomenon, the timing, and the cell target. Eventual antiapoptotic therapies should be targeted to a particular cell type, lethal stimulus, and time frame as narrowly as possible.

tubular cell death in the pathogenesis of AKI includes the fact that several nephrotoxins that induce AKI also promote tubular cell death in culture, that therapeutic intervention on apoptosis improves experimental AKI, and that a bioartificial kidney containing proximal tubular cells improves survival in experimental animals and, in preliminary studies, in humans. Tubular cell death in the early stages of AKI of different etiologies (ischemic, toxic, septic, obstructive) can proceed through apoptosis or necrosis. The relative contribution of the two mechanisms to tubular cell loss depends on the severity of the insult. A second peak of apoptosis occurs days (it peaks at day 8 in rat ischemic AKI) after the original insult, when the injured tubules have been completely reconstituted by a hyperplastic epithelium. In this case, apoptosis restores cell number to preinjury levels.

In CKD, progressive loss of renal mass and function leads to end-stage renal disease, necessitating replacement of renal function by dialysis or transplantation. The personal, social, and economic costs of these therapies are staggering at approximately 20 billion US dollars per year in the United States. Apoptotic cell death exceeding mitotic replacement contributes to renal cell loss in the form of podocytopenia, glomerulosclerosis, and tubular atrophy. This has been documented for podocytes and mesangial, endothelial, and tubular cells in experimental models of progressive glomerular scarring and tubulointerstitial atrophy. Consistent with the experimental data, an increased rate of apoptosis has been observed in human CKD.

Diabetic nephropathy, vascular injury, glomerular injury, and PKD are frequent causes of CKD. Diabetic nephropathy is the most common cause of end-stage renal disease. Hyperglycemia is the primary metabolic alteration that promotes diabetic tissue injury. However, glucose degradation products and elevated local cytokine (e.g., tumor necrosis factor [TNF], TNF-related apoptosis-inducing ligand [TRAIL]; TGF β 1, angiotensin II) levels also contribute to tissue injury and renal cell apoptosis (Figure 22-1). The glomerulus was long thought to be the primary site of injury in diabetic nephropathy. Recently, podocytopenia was identified as an early feature of diabetic nephropathy and podocyte apoptosis as a primary contributor. In addition, tubular cell apoptosis is prominent in diabetic nephropathy. The expression of 112 cell death-related genes was abnormal in the tubulointerstitium of diabetic nephropathy patients, and diabetic individuals are sensitized to AKI. One hypothesis attributes the sensitization to AKI to an abnormal pattern of apoptotic gene expression that favors renal cell death.

Other forms of glomerular injury are usually the result of immune or inflammatory aggression. Inflammatory mediators may cause mesangial, glomerular endothelial cell, and podocyte apoptosis, ultimately leading to glomerular scarring (glomerulosclerosis). TGF β 1, angiotensin II, a high glucose concentration, mechanical stress (which may result from increased single-nephron glomerular filtration rate in remnant

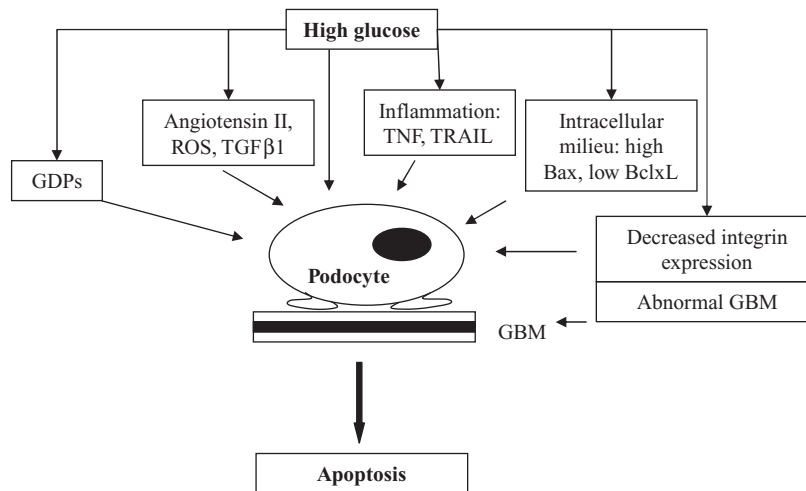


Figure 22-1. Factors contributing to renal cell apoptosis in diabetic nephropathy, as exemplified by the podocyte. High glucose concentrations induce renal cell apoptosis. In addition, they promote the release of ROS, angiotensin II, and TGF β 1 and disturb the intracellular milieu and the expression of cell membrane receptors. The inflammatory milieu of the diabetic kidney may also induce apoptosis. Glycation of extracellular matrix proteins as well as an abnormal pattern of extracellular matrix deposition changes the composition of the GBM. Finally, glucose breakdown products may also have direct proapoptotic activity. Similar mechanisms are active in tubular cells. GBM, glomerular basement membrane; GDPs, glucose degradation products; ROS, reactive oxygen species.

nephrons), and death receptors induce apoptosis of cultured glomerular cells.

Renal ischemia is the primary pathogenic mechanism in renal vascular injury. Ischemia as a contributor to renal cell apoptosis has been usually studied in cell models or animal models of AKI.

Human PKD is a group of hereditary diseases caused by mutations in genes that encode primary cilia proteins. The most frequently mutated gene is *pkd1*. PKD is characterized by increased tubular proliferation, apoptosis, and dedifferentiation, leading to the growth of fluid-filled cysts in kidneys. No effective treatment is currently available. *Bcl-2*^{-/-} mice develop polycystic kidneys, but the mechanisms differ from those of *pkd1*^{-/-} mice in that disease in the latter is not prevented by absence of Bim. At present, the relationship between uncontrolled proliferation and apoptosis has not been clarified. Both caspase inhibitors and the cell cycle inhibitor roscovitine decrease proliferation and apoptosis and prevent disease progression in animal models.

4. REGULATION OF APOPTOSIS IN KIDNEY CELLS

Renal cell death is usually a response to the cell microenvironment. The absence of certain factors (survival factors) or the presence of lethal factors promotes apoptosis. Surrounding cells, soluble mediators, and the extracellular matrix regulate cell death and survival. Surrounding cells can synthesize survival

or lethal factors or compete for such factors.

4.1. Survival factors

Survival factors for tubular and mesangial cells and podocytes include insulin-like growth factor 1 (IGF-1), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), GDNF, erythropoietin, and parathyroid hormone-related protein. These factors activate the PI3K/AKT survival pathway. AKT has several antiapoptotic actions. AKT phosphorylates and inhibits proapoptotic factors such as BAD. Unphosphorylated BAD binds to BclxL, blocking its survival function. BAD dephosphorylation is observed in tubular cells exposed to proapoptotic stimuli.

Cultured tubular cells exposed to survival factors develop a general intracellular milieu favoring survival that

includes low levels of proapoptotic Fas receptor and Bax and higher levels of antiapoptotic BclxL and Bcl2. In addition, compensatory survival pathways are activated in the course of injury that may limit the extent of injury and accelerate recovery. AKT is activated in tubular cells after renal ischemia/reperfusion injury, tubular cell BclxL is increased in experimental AKI, and VEGF is increased in cyclosporin A (CsA) nephrotoxicity in vivo and in cultured tubular cells. Podocyte Hsp27 is increased in rat models of podocyte injury. Over-expression of Hsp27 in cultured podocytes preserved morphological features and improved podocyte survival. The GDNF receptor tyrosine kinase, RET, was upregulated in podocytes in experimental proteinuric nephropathies and in cultured mouse podocytes after injury induced by sublytic C5b-9. GDNF and VEGF are also induced during podocyte injury and behave as autocrine survival factors. Clusterin (SPG40) is a multifunctional protein whose expression is increased in renal injury and protects cells from apoptosis in vitro and in vivo. Exogenous clusterin prevents peroxide hydrogen cytotoxicity in tubular cells. Interference with these compensatory mechanisms aggravates renal injury as exemplified by the more severe renal injury when endogenous VEGF is neutralized in CsA nephrotoxicity. Therapeutic interventions designed to potentiate endogenous adaptive pathways have been successfully employed in experimental AKI and CKD. IGF-1, HGF, erythropoietin, and non-erythropoietic erythropoietin-like molecules have

prevented experimental AKI, decreasing apoptosis and improving renal function.

Attachment to a normal basement membrane will prevent anoikis in podocytes and tubular cells. Some disease processes are characterized by hereditary (e.g., Alport syndrome caused by mutations in type IV collagen genes) or acquired (e.g., diabetic nephropathy) alterations of basement membrane matrix proteins. An altered basement membrane may contribute to reduced podocyte survival, and similar processes may be operative for tubular cells. The recovery of live podocytes from urine suggests that detached podocytes may be rescued from anoikis if an appropriate microenvironment is restored. Extracellular matrix proteins activate survival mechanism dependent on focal adhesion kinase and Ras-ERK signaling pathway. In addition to basement membrane changes, podocyte or tubular cell injury may lead to impaired function of receptors for extracellular matrix proteins. In podocytes, $\alpha 3 \beta 1$ integrin, α -actinin-4, and the dystroglycan complex are required for podocyte survival by facilitating adhesion to the glomerular basement membrane. $\alpha 3 \beta 1$ integrin is decreased in podocytes from humans and rats with diabetes, and high glucose media decreases the expression of $\alpha 3 \beta 1$ integrin via TGF β in cultured podocytes. During AKI injured tubular cells lose polarity, dedifferentiate, and detach. The requirement for a normal extracellular matrix is also observed in mesangial cells. Laminin protects rat mesangial cells from apoptosis induced by serum starvation and DNA damage by a $\beta 1$ integrin-mediated mechanism.

Novel relevant antiapoptotic molecules for renal cells have been recently identified. Cyclin I has a survival role in podocytes. By binding to CDK5, cyclin I increases BclxL and Bcl2 expression and decreases BAD expression. Cyclin I knockout podocytes were more susceptible to apoptosis both in vitro and in vivo through stabilization of p21. In addition, the CDK2 inhibitor p27kip1 has been related to apoptosis. Indeed, p27kip1^{-/-} mice develop more intense tubular apoptosis after ureteral obstruction as well as more severe glomerulonephritis. Survivin, an inhibitor of apoptosis protein, has recently been identified as a constitutive prosurvival molecule in tubular cells that protects from experimental AKI.

4.2. Lethal factors

Cytokines, ischemia, endogenous toxic metabolites, or exogenous toxins may cause renal cell death in the complex environment of the injured kidney. Cytokines and hyperglycemia may induce apoptosis of glomerular and tubular cells. However, the main target of

ischemia-reperfusion injury and xenobiotics are tubular cells, especially proximal tubular cells, as a result of the presence of transporters that favor the intracellular accumulation of toxins and the high number of mitochondria to fuel molecular transport. Endothelial cells have been less studied, but they may also succumb to cytokines, ischemia/reperfusion toxic metabolites, and xenobiotics.

4.2.1. TNF superfamily cytokines

TNF α , FasL, TRAIL, and TNF-like weak inducer of apoptosis (TWEAK) can induce, depending on the microenvironment, apoptosis of mesangial cells, tubular epithelial cells, podocytes, and renal endothelial cells. The importance of cooperation between lethal factors has been underscored by the analysis of complex biological systems. Changes in the level of expression or activation of apoptosis regulatory molecules may explain the cooperation of cytokines in inducing cell death. As an example, TNF α increases the expression of TWEAK receptor, Fas, Bax, and Smac/DIABLO while decreasing that of BclxL in tubular epithelium. In tubular cells, TNF α -induced apoptosis is facilitated by deprivation of survival factors. FasL requires the upregulation of Fas receptor expression by survival factor deprivation or by the presence of an inflammatory milieu. By contrast, Fas activation induces death in nonstimulated mesangial cells in vitro and in vivo. TWEAK alone induces mesangial cell apoptosis, but not tubular cell death, that requires the concomitant presence of TNF α and interferon- γ (IFN γ). TRAIL is the most upregulated TNF superfamily gene in diabetic nephropathy tubulointerstitium. TRAIL is more lethal for tubular cells in a high-glucose inflammatory milieu. However, the most studied lethal cytokine is FasL. A number of apoptotic factors or settings involved in the pathogenesis of renal injury upregulate Fas expression in renal cells, and at least some of them render the cells more susceptible to FasL-induced apoptosis: cytokines (TNF γ , IFN γ , interleukin [IL] 1 β , IL-1 α), bacterial lipopolysaccharide (LPS), nephrotoxins, HIV infection, and deprivation of survival factors (Figure 22-2). Plasma from patients with thrombotic microangiopathy induces apoptosis and Fas expression in renal microvascular endothelial cells. In mesangial and tubular epithelial cells, protein synthesis inhibitors induce apoptosis and also sensitize to apoptosis mediated by the death receptors TNFR and Fas; these findings suggest that ongoing synthesis of protective proteins is required to prevent programmed cell death.

Tubular Fas-associated death domain protein (FADD) is upregulated in experimental AKI. FADD-DD is a

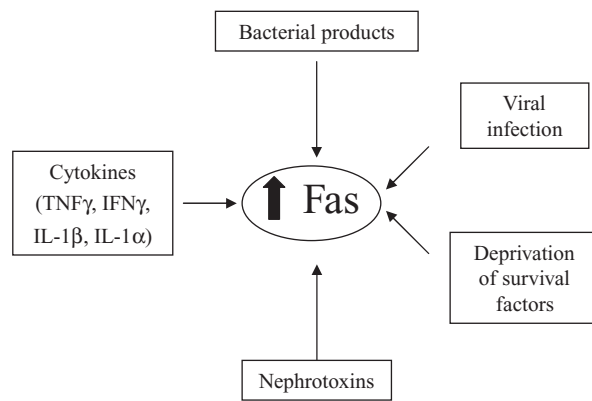


Figure 22-2. The microenvironment modulates the sensitivity of renal cells to lethal stimuli. The influence of microenvironmental factors on the expression of Fas and the sensitivity to FasL-induced death has been extensively studied in renal cells. Inflammation (cytokines), viral (HIV) or bacterial (LPS) infection, nephrotoxins (cyclosporin A, acetaminophen), and deprivation of survival factors increase Fas expression in renal cells, and, with the exception of nephrotoxins, sensitivity to FasL.

truncated molecule corresponding to the death domain (DD) of FADD that behaves as a FADD antagonist in some cell systems. Surprisingly, in tubular cells, FADD-DD is sufficient to promote a caspase-independent form of cell death. This is consistent with a role for FADD in death receptor-independent events.

4.2.2. Other cytokines

Several cytokines may induce apoptosis by triggering the intrinsic pathway of apoptosis independently of death receptors. Two key mediators of renal injury, TGF β 1 and angiotensin II, may induce apoptosis in renal tubular epithelial cells and podocytes.

The expression of TGF β 1 and its receptors is increased in a variety of glomerular diseases characterized by podocyte injury and proteinuria, including membranous nephropathy, diabetic nephropathy, and focal segmental glomerulosclerosis. Podocytes secrete TGF β 1 in response to several agents, such as high glucose, low-density lipoprotein (LDL), or thrombin. TGF β 1-induced apoptosis requires activation of p38 MAP kinase and engages several downstream mediators. SMAD-7, Bax synthesis, and caspase-3 activity are increased in TGF β 1-induced apoptosis. The proapoptotic effects of Smad7 over-expression and of TGF β 1 are additive. However, unlike TGF β 1, Smad7 inhibits the nuclear translocation and transcriptional activity of the cell survival factor nuclear factor kappa B (NF- κ B). The cyclin-dependent kinase inhibitor p21 is also increased in podocytes in experimental membranous nephropathy and diabetic nephropathy models. TGF β 1 increases p21 levels in

cultured podocytes and, in turn, p21 prevents the compensatory upregulation of antiapoptotic Bcl-2 that takes place under disease conditions to improve the chances of survival. The fact that p21-null podocytes were protected from TGF β 1-induced apoptosis supports a critical role for p21 in TGF β 1-induced apoptosis. In addition, TGF β 1 impairs the adhesion to the glomerular basement membrane by downregulating the expression of α 3 β 1 integrin. TGF β 1 may also induce tubular cell apoptosis and epithelial-mesenchymal transition.

Angiotensin II is a mediator of stress tension (induced by mechanical stretch)-induced podocyte apoptosis and directly causes podocyte apoptosis through activation of the AT1 receptor.

4.2.3. Glucose

Besides the proapoptotic actions of cytokines expressed in diabetic tissues, hyperglycemia directly induces apoptosis in cultured podocytes and tubular cells (Figure 22-1). Glucose may also sensitize to cell death induced by other stimuli by upregulating Bax and Basp1 and downregulating BclxL in tubular cells. A further mechanism of podocyte loss in diabetes may relate to the detachment of podocytes from an abnormal glomerular basement membrane. Activation of poly (ADP ribose) polymerase (PARP) plays an important role in the pathophysiology of various diseases associated with oxidative stress, such as diabetes. PARP inhibitors blocked hyperglycemia-induced podocyte apoptosis in vitro. In addition, glucose degradation products, such as 3,4-dideoxyglucosone-3-en (3,4-DGE), induce Bax-dependent apoptosis in tubular cells and podocytes.

Other agents whose role in glomerular injury is less well characterized also promote renal cell apoptosis. Oxidized LDL induced apoptosis in human cultured podocytes by reducing Akt activity. Reactive oxygen species themselves promote apoptosis of renal cells.

4.2.4. Drugs and xenobiotics

There are multiple nephrotoxic drugs. However, for some of them, nephrotoxicity is the dose-limiting side effect. Examples include the immunosuppressant CsA, the aminoglycoside antibiotics, the antifungal amphotericin B, the antiviral cidofovir, and the antineoplastic cisplatin. All of them may cause AKI and CKD. In addition, acetaminophen overdoses may cause AKI. The study of the molecular mechanisms engaged by nephrotoxins that induce AKI and cultured tubular cell apoptosis has disclosed stimulus-specific pathways that may lead to specific interventions (Figure 22-3).

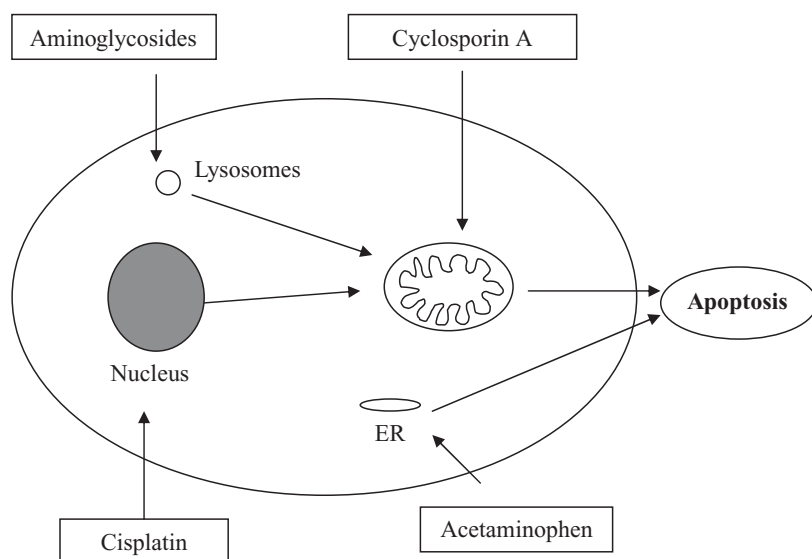


Figure 22-3. The study of the lethal molecular pathways engaged by nephrotoxins has uncovered examples of stimulus-specific apoptosis pathways in renal tubular epithelial cells. Cyclosporin A induces Bax-mediated mitochondrial injury. Acetaminophen induces mitochondria-independent endoplasmic reticulum (ER) injury. Aminoglycosides accumulate in lysosomes. Their eventual release (and probably of other lysosomal contents) activates the mitochondrial pathway for cell death. DNA injury activates p53-mediated apoptosis in cisplatin nephrotoxicity.

CsA increases Fas expression in tubular cells in culture and in vivo. However, neither neutralizing anti-FasL antibodies nor caspase-8 inhibitors decreased apoptosis induced by CsA. Similar observations were made for acetaminophen. This suggests that some changes in apoptosis-related molecules are epiphenomena not directly related to cell death. By contrast, Bax-mediated mitochondrial injury and caspase activation are key events in CsA-induced apoptosis of tubular cells. CsA induces Bax aggregation and translocation to mitochondria, causing mitochondrial outer membrane permeabilization, release of cytochrome c and Smac/DIABLO, and activation of caspases-9 and -3. Initiator caspase-2 is also activated and may lead to mitochondrial injury. In a positive feedback loop, caspases further damage the mitochondria, leading to loss of mitochondrial transmembrane potential. The feedback loop is essential for apoptosis and cell death to proceed because caspase inhibitors prevented both. This is one of several models for the participation of mitochondrial injury in apoptosis. Bax antisense oligodeoxynucleotides prevent CsA-induced apoptosis. Bax is also required for apoptosis and cell death induced by 3,4-DGE, a toxic glucose metabolite. CsA is a potent inhibitor of macrophage apoptosis through the inhibition of inducible nitric oxide synthase, illustrating cell-specific pathways.

Acetaminophen induces caspase-dependant apoptosis of tubular cells without characteristic mitochondrial alterations or Bax involvement. Acetaminophen

nephrotoxicity appears to be an example of the involvement of the endoplasmic reticulum (ER) in apoptosis. ER-initiated apoptosis may be triggered by disturbances of calcium homeostasis or accumulation of misfolded proteins, and multiple signaling pathways emerge to promote cell death via caspase-dependent and -independent means, including the recruitment of the mitochondrial pathway. Molecular responses characteristic of involvement of the ER in apoptosis include the expression of C/EBP homologous protein (CHOP)/GADD153, a transcription factor that decreases Bcl-2 levels, and activation of ER-associated caspase-12. Caspase-12 is present in mice, but most humans carry an inactivating mutation. Acetaminophen upregulated CHOP/GADD153 and lead to caspase-12 cleavage and apoptosis in tubular cells.

Caspase inhibition protected tubular cells from acetaminophen-induced apoptosis, but not from eventual cell death. By contrast, BclxL protected tubular cells from death. BclxL interacts with several ER proteins. CsA increased CHOP/GADD153 expression but failed to activate caspase-12, suggesting that CHOP upregulation may be induced by non-ER stressors. The ER stressor tunicamycin induced severe histological tubular injury, which was decreased both in CHOP/GADD153 and caspase-12 knockout mice. Although these studies serve as a proof of concept for the relevance of ER stress in tubular injury, tunicamycin has no direct clinical relevance. In a more clinically relevant model, ischemia/reperfusion, ORP150 (150-kDa oxygen-regulated protein), an inducible ER chaperone, was upregulated in tubular epithelium and shown to protect from ischemia/reperfusion or hypoxia.

Aminoglycoside nephrotoxicity is an example of lysosomal participation in apoptosis. Lysosomal accumulation of gentamicin may initially prevent its more toxic cytosolic localization. Eventually, lysosomal membrane permeabilization releases free gentamicin to the cytosol and/or releases other lysosomal components that trigger a Bax-mediated mitochondrial pathway of apoptosis.

The proapoptotic role of p53 has been characterized in cisplatin nephrotoxicity. Cisplatin damages genomic DNA and markedly induces p53 expression and phosphorylation. Pifithrin- α inhibits transcriptional and nontranscriptional activities of p53 and protects tubular cells in culture and in vivo. p53

transcriptional targets include TRAIL receptors, Noxa, Bax, p53-upregulated modulator of apoptosis (PUMA), and p53-induced protein with a death domain (PIDD). The expression of the latter two is critical for p53 nephrotoxicity. PUMA antagonizes Bcl-xL. PIDD promotes the formation of a multiprotein complex, the PIDDosome, leading to caspase-2 activation, which causes the release of AIF from mitochondria. Inhibition of p53, caspase-2, or apoptosis-inducing factor (AIF) markedly protected against cisplatin-induced apoptosis in cultured tubular cells. p53 nontranscriptional actions include inactivating Bcl2/Bcl_{xL} and activating Bax. In addition, cisplatin activates mitogen-activated protein kinases. In the context of cisplatin nephrotoxicity, extracellular signal-regulated kinase (ERK) promotes apoptosis, contrary to its usual role in cell death regulation. Cdk2 and E2F1 also participate in cisplatin-induced tubular cell death.

Puromycin aminonucleoside (PAN), a drug commonly used to induce experimental nephrotic syndrome in rats, also induces podocyte apoptosis. PAN-induced podocyte apoptosis is mediated by ROS, Bax, p53, and AIF. However, PAN does not induce proteinuria in mice and is not in clinical use.

4.2.5. Ischemia-reperfusion and sepsis

Ischemia-reperfusion is a frequent cause of AKI, especially in renal transplantation and intensive care units. Mitochondria, death receptors, p53, caspases, and ER stress have all been implicated by interventional studies in tubular cell death after ischemia-reperfusion. In this model, Bid connects the death receptor and mitochondrial pathways. In the intensive care setting, renal ischemia-reperfusion usually coexists with other causes of AKI, such as sepsis and nephrotoxins. Multiple cytokines contribute to renal injury in sepsis. Bacterial LPS itself increases Bak and downregulates Bcl_{xL}, inducing apoptosis in glomerular endothelial cells and upregulating Fas in tubular and mesangial cells.

5. THERAPEUTIC APPROACHES

Some of the drugs currently in use for CKD or glomerular injury have been recently shown to target renal cell apoptosis, besides having other beneficial effects (Table 22-3). In addition, new drugs targeting apoptosis are under development. The characterization of the molecular pathways activated at each stage of renal injury, the cell targets, and the time frames will be crucial to develop sensible therapeutic strategies. Although lethal factors result in tissue injury, it is commonly thought that competition for survival factors is a key determinant of survival during the second compensatory wave

Table 22-3. Therapy targeting apoptosis in kidney injury

Drugs or drug targets	Current indication
New tricks for old drugs	
ACEI/ARB	CKD, hypertension, proteinuria, glomerular injury, diabetic nephropathy
Steroids	Nephrotic syndrome, glomerulonephritis
Erythropoietin	Uremic anemia
Darbepoetin	Uremic anemia
Statins	Hypercholesterolemia
New drugs or targets	
Lethal cytokines	—
Survival factors	—
Caspase inhibitors	—
BH4-like	—
Basp1	—
Bax inhibitors	—
Pifithrin- α	—
<i>Note:</i> Recent research has identified inhibition of renal cell apoptosis as a mechanism of action of some drugs currently used in nephrology. In addition, some novel approaches have been successful in animal or cell models. ACEI, angiotensin-converting enzyme inhibitors; ARB, angiotensin receptor blockers.	

of apoptosis, leading to disappearance of hyperplasia and recovery from AKI. Given the potential of apoptosis modulation to interfere with physiologic apoptosis, the most likely initial clinical translation of antiapoptotic drugs will be processes in which there is limited systemic exposure to the drug or the drug is administered during a short time period. Prevention of AKI will be the most likely objective for novel antiapoptotic drugs. Inclusion of antiapoptotic drugs in solutions used to preserve organs for transplantation with the aim of reducing ischemia-reperfusion injury will limit drug exposure in time and space. In addition, short-term prophylactic administration in situations in which AKI is highly likely may be explored. Such situations include extracorporeal circulation cardiac surgery or administration of nephrotoxic drugs such as cidofovir. Nephrotoxicity is the dose-limiting effect of this antiviral drug, which is administered iv every 2 weeks, thus facilitating prophylactic intervention. In the following discussion, we focus on approaches that directly target apoptosis, skipping alternative therapeutic approaches such as decreasing access of nephrotoxins to tubular cells and other maneuvers. Small molecules may be bound to carriers that lead to specific proximal tubular uptake and organ protection.

Among currently used drugs that also target apoptosis, we find angiotensin-converting enzyme inhibitors (ACEIs), angiotensin type 1 receptor blockers (ARBs), steroids, and erythropoietins.

ACEIs and ARBs are antihypertensive drugs used to treat CKD when proteinuria is present. In addition to reducing systemic and intraglomerular pressures, angiotensin II blockade decreases podocyte apoptosis induced by either angiotensin II or mechanical stretch. Corticosteroids are immunosuppressive drugs that have long been used to treat proteinuric kidney disease of immune origin and are the mainstay of therapy of minimal-change nephrotic syndrome. Dexamethasone markedly reduces apoptosis in cultured podocytes by decreasing p53, increasing Bcl-xL, and inhibiting AIF translocation. Erythropoietin and darbepoetin are used in CKD patients for the treatment of renal anemia. They also have antiapoptotic and tissue-protective actions. Darbepoetin protects podocytes from sublethal injury and apoptotic cell death. Ongoing clinical trials are exploring the role of erythropoietin in prevention of AKI after kidney transplantation. Statins are frequently used in proteinuric patients to lower LDL cholesterol levels. Experimental animal and cell culture studies suggest that statins inhibit cultured podocyte apoptosis by stimulating Akt activity. It is interesting to note that lovastatin induces apoptosis in actively proliferating mesangial cells and spares quiescent cells grown in serum-free conditions. This property could be used therapeutically to target proliferating mesangial cells in vivo. However, statins also induce apoptosis in proliferating tubular cells.

Among potential novel targets we find growth factors, cytokines, Bcl2-like proteins, caspases, and p53. Survival factors and anti-cytokine strategies have been used to prevent apoptosis in animal models, but clinical trials have not been performed, or, in the case of IGF-1 for AKI, have failed to demonstrate benefit. A decrease in BclxL levels is a common event in tubular cell death induced by different mechanisms. BclxL over-expression protected tubular cells from apoptosis induced by acetaminophen, CsA, and death receptors. More recently, the cell-permeable BclxL-like molecule TAT-BH4 containing the BH4 domain of BclxL fused to the protein transduction domain of HIV TAT has efficiently prevented apoptosis in cultured cells and in vivo. A KU-70–derived Bax-targeting peptide afforded protection in tubular cell culture studies.

In vivo caspase inhibitors protect against ischemic injury in kidney. The pan caspase inhibitor zVAD prevented renal function impairment at an early time point (24 hours) when administered at the time of reperfusion. It was much less effective when administered 2 hours later. Longer follow-up studies are needed to exclude the possibility that zVAD is only retarding cell death and favoring more injurious necrotic cell death. In

this regard, zVAD exacerbated TNF α toxicity by enhancing oxidative stress and mitochondrial damage, resulting in hyperacute hemodynamic collapse, kidney failure, and death. In tubular cells exposed to TWEAK, TNF α , and IFN γ , inhibition of caspase-8 or multiple caspases transformed a weak apoptotic response into massive reactive oxygen species–dependent necrosis. In addition to their role in apoptosis, caspases have also nonapoptotic roles in inflammation, cell proliferation, and differentiation that may complicate their therapeutic targeting. Thus interference with inflammation via IL-18 was instrumental in protection against ischemia-reperfusion injury afforded by caspase-1 deficiency or inhibition.

Basp1 was recently shown to be required for high glucose-induced apoptosis in tubular cells and Basp1 targeting by siRNA was protective.

The small-molecule p53 inhibitor pifithrin- α prevented apoptosis and protected renal function in experimental ischemia-reperfusion and cisplatin nephrotoxicity.

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23

Physiologic and Pathological Cell Death in the Mammary Gland

Armelle Melet and Roya Khosravi-Far

1. INTRODUCTION

Apoptosis is a regulated cell suicide program that functions to control mitosis during the development and maintenance of tissues. This type of cell death plays a key role in the extensive postnatal development of the breast. Dysregulation in apoptosis has been speculated to contribute to hyperplasia and to promote breast cancer and resistance to chemotherapy. Recent studies have highlighted that other modes of cell death additionally influence breast development, tumorigenesis, or response to chemotherapy. This chapter reviews the role and regulation of apoptosis in the normal and neoplastic breast. It also briefly summarizes the contribution of other types of cell death, such as autophagy, necrosis, or entosis.

2. APOPTOSIS IN THE NORMAL BREAST

2.1. Occurrence and role of apoptosis in the developing breast

The breast is a hormone-responsive organ that undergoes major functional and morphological changes postnatally, during puberty, and during pregnancy. Extensive studies in animal models have established that apoptosis plays a critical role in these physiologic processes. Apoptotic cell death occurs mainly in the breast epithelium, which develops gradually into hollow tree-like structures (ducts and terminal alveoli) surrounded by fatty, fibrous, and glandular connective tissues (the stroma).

At birth, the mammary gland consists of a rudimentary ductal network protruding from the nipple into the stromal fat pad (Figure 23-1A). The ductal network expands and arborizes mostly at puberty. The ovarian hormone estrogen and the pituitary growth hormone stimulate

the growth and branching of highly proliferative bulbous structures at the ductal tips called the terminal end buds (TEBs) (Figure 23-1B). The TEBs are composed of two distinct cell types, the cap and body cells, which are the progenitors of the outer myoepithelium and the lumen epithelium, respectively. The luminal body cells undergo extensive detachment-induced apoptosis (anoikis) to hollow out the elongated part of the duct. When the expanding ductal branches reach the limits of the mammary fat pad, the TEBs differentiate and are permanently replaced by terminal end ducts or alveolar buds, with this alveolar differentiation starting as sexual maturity is reached.

Apoptosis not only occurs during ductal morphogenesis, but also takes place in mature females to maintain tissue homeostasis. During the menstrual/estrous cycle, the adult mammary gland responds to systemic hormonal changes by cycles of limited proliferation, differentiation, and apoptosis in a small subset of epithelial cells. Thereby, the mammary gland prepares for a possible pregnancy with a modest development of alveolar structures and regresses by apoptosis in the absence of pregnancy. The frequency of apoptosis fluctuates with steroid hormones levels, with a peak of apoptosis following a peak of proliferation close to the end of the menstrual cycle.

The final differentiation of the mammary gland only takes place during pregnancy and lactation. Pregnancy hormones (estrogen, progesterone, and prolactin) induce the shrinking of stromal adipocytes, additional ductal branching, and further growth and differentiation of the alveoli into milk-secreting lobules (the mammary acini). During lactation, the functional and morphogenetic development of the breast is fulfilled and apoptosis is inhibited. The milk produced in the lobuloalveolar structures can then be expelled thanks to the

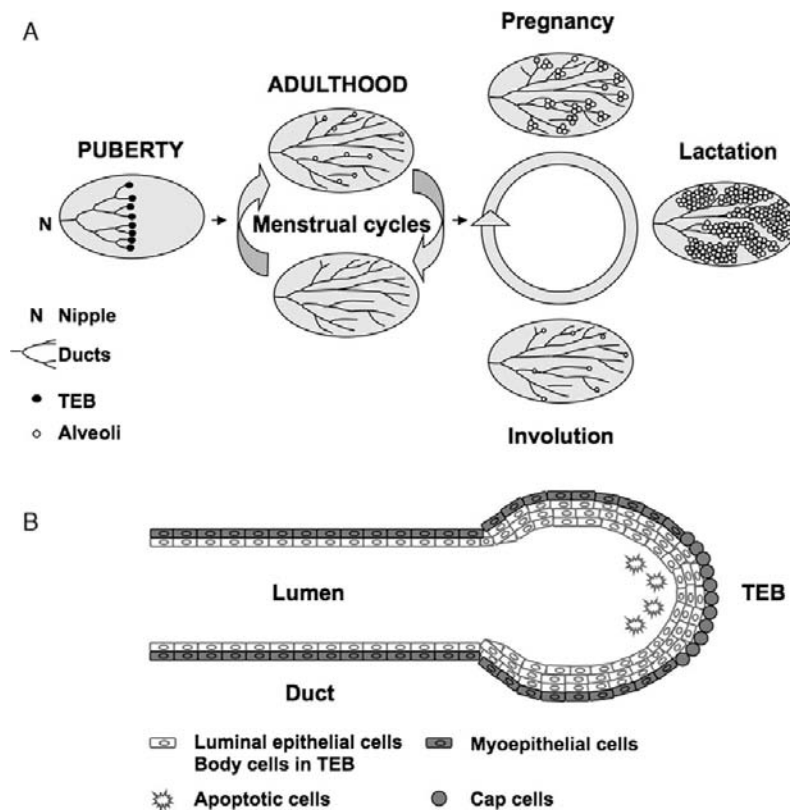


Figure 23-1. Apoptosis during the postnatal development of mammary gland. **(A)** Schematic overview of mammary gland development. Major events of cell proliferation, differentiation, and apoptosis take place in the mammary epithelium during the postnatal development of the breast. During puberty, estrogen and growth hormone promote ductal outgrowth. The ends of the ducts form highly proliferative bulbous structures called terminal end buds (TEBs). These TEBs expand and hollow out by anoikis of luminal cells. In the mature female, the entire fat pad is filled with a tree-like network of branching ducts. Cyclic hormonal changes stimulate a modest development of alveoli that are eliminated by apoptosis in the absence of pregnancy. Pregnancy hormones (progesterone, prolactin, and placental lactogens) induce the growth of alveoli that differentiate into milk-secreting alveoli at the end of pregnancy. During lactation, luminal cells of mature alveoli secrete milk to feed the newborn. When lactation ceases, the majority of the secretory epithelium is removed by apoptosis during involution. The oval shapes depict the mammary fat pad (the stroma); the other labels are shown directly on the figure. **(B)** Schematic representation of the terminal end buds (TEBs). TEBs are highly proliferative bulbous structures that appear at the ductal tips at puberty. TEBs are composed of two types of stem cells: the outer cap cells and several layers of inner body cells. These two cell types are the progenitors of the ductal myoepithelium and the lumen epithelium, respectively. The central luminal body cells undergo detachment-induced apoptosis (anoikis) to clear the lumen of the elongating ducts during ductal morphogenesis. Labels are shown directly on the figure.

contractile myoepithelium and transported to the nipples through the luminal space of the ductal network. By clearing the lumen of the developing ducts and alveoli, apoptosis is thus essential to the breast's ultimate function (i.e., milk production and secretion).

When lactation ends, the secretory epithelium undergoes massive apoptosis, and the gland remodels to a quiescent state in a two-phase involution process. The first apoptotic phase is triggered by milk stasis and is reversible by suckling up to 48 hours in mice. The second, irreversible phase is driven by the drop

in lactogenic hormones and involves proteolytic degradation of the basement membrane, further detachment-induced apoptosis (anoikis), collapse of alveoli, and tissue remodeling. The involution process, which removes the majority of the secretory epithelium, constitutes the most dramatic occurrence of apoptosis in the normal breast.

Finally, after menopause, the aging mammary gland undergoes lobular involution through unknown mechanisms, probably combining epithelial apoptosis and senescence.

This overview of mammary development highlights the critical role of apoptosis in morphogenesis and homeostasis of the normal breast (Figure 23-1). Two major events of apoptosis take place in the epithelium of the mammary gland. First, during puberty and pregnancy, apoptosis contributes to shape the lumen of the developing ducts and alveoli. Second, during involution, post-lactational milk stasis triggers an extensive wave of apoptosis that removes the excess milk-secreting epithelium. Given its importance for the development and function of the breast, apoptosis is tightly regulated by both intracellular and extracellular factors.

2.2. Molecular regulation of apoptosis in the normal breast

The mammary gland is a complex organ with a highly organized cellular architecture composed of epithelial cells and stromal cells (fibroblasts, adipocytes, immune and inflammatory cells, endothelial cells) that communi-

cate with each other via an extracellular matrix (ECM) through adhesive connections and soluble secreted factors. Three-dimensional (3D) cell culture and in vivo animal models have been very useful to recapitulate the complexity of the 3D cellular organization and interactions in this organ. These models were used to identify the key components of apoptotic regulation in the mammary gland, those being derived from both the epithelial cells and their complex surrounding microenvironment. Apoptosis of mammary epithelial cells is thus regulated at three levels, by intracellular regulators (i.e., BCL-2),

by local mammary factors (autocrine/paracrine secreted factors, ECM, and cell adhesion proteins), and finally by systemic hormones that regulate the expression and activity of the latter.

Ovarian steroid hormones (estrogen, progesterone) and pituitary peptide hormones (prolactin) are potent inhibitors of apoptosis in hormone-responsive tissues such as the breast. Decline in these systemic hormone levels is associated with apoptosis and regression of the mammary gland during involution. This hormonal control of apoptosis has been extensively described elsewhere. This review focuses on the local and intracellular regulation of apoptosis in the mammary gland, with major emphasis on the involution phase.

Involution has been used as a model to study apoptosis regulation in the mammary gland. At the onset of involution, milk accumulates locally within alveolar lumens, while systemically, levels of lactogenic hormones fall. Local milk accumulation (and not systemic hormones) appears to be the critical apoptotic inducer. Indeed, in a mouse model of lactation failure, the artificial addition of lactogenic hormones does not affect apoptosis, although it prevents the remodeling of the involuting gland. The first phase of involution is therefore initiated locally by mammary-derived factors. The precise initial trigger of apoptosis in the involuting breast is currently unknown, but two hypotheses prevail. Apoptosis could be triggered by an accumulation of apoptosis-inducing factors in the milk and/or by a physical distortion of secretory epithelial cells generated by the engorgement. Subsequently, an interplay of different signaling pathways is activated to induce apoptosis in the mammary gland (Figure 23-2). Microarray analyses show that the two stages of involution are controlled by a temporal change in gene expression with progressive gain of death signals and loss of survival factors (Table 23-1). Apoptosis in the first phase involves both death receptor and mitochondrial pathways, whereas apoptosis/anoikis in the second phase is most likely mediated by the classic

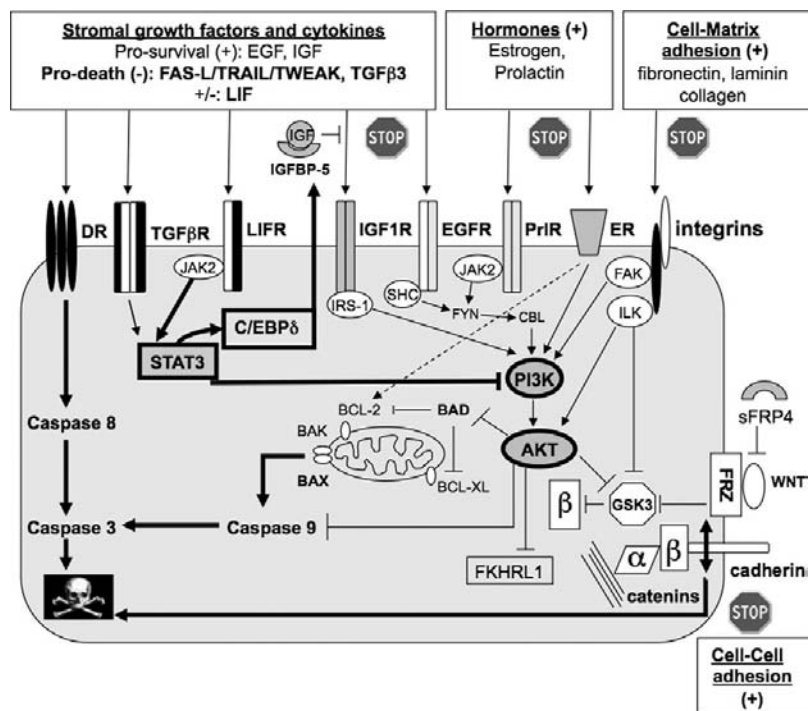


Figure 23-2. Death signaling pathways in mammary epithelial cells during involution. Apoptosis of mammary epithelial cells is controlled by systemic hormones, stromal growth factors and cytokines, and cell–cell and cell–matrix adhesion. Apoptotic death during involution is triggered by a combined gain of death signals and loss of survival signals. Main death pathways involved in involution are shown in bold. Transcription factors are symbolized by rectangles within the epithelial cell figure. Local milk stasis causes the stretching of alveoli, leading to the disruption of prosurvival cell–matrix and cell–cell adhesions. Along these lines, truncation of the β -catenin binding domain of E-cadherin was shown to precede epithelial apoptosis in early mammary involution. Milk stasis also induces the expression of several proapoptotic cytokines (LIF, TGF β 3, death ligands) that trigger apoptosis through the extrinsic death receptor pathway and the STAT3 pathway. Survival pathways such as the IGF and the PI3K/AKT signaling are inhibited, and proapoptotic members of the BCL-2 family such as BAD and BAX are upregulated to activate the mitochondrial intrinsic death pathway. (+) prosurvival signals which are inhibited during involution (stop signs) and (–) prodeath signals. DR, death receptor; TGF β R, transforming growth factor β receptor; LIFR, leukemia inhibitory factor receptor; IGF1R, insulin-like growth factor receptor type 1; EGFR, epidermal growth factor receptor; PrIR, prolactin receptor; ER, estrogen receptor; FRZ, frizzled receptor; sFRP4, secreted frizzled-related protein 4; GSK3, glycogen synthase kinase 4.

mitochondrial pathway. Key molecules and pathways controlling apoptosis in the normal breast are described below.

2.2.1. Autocrine/paracrine regulation by growth factors, death ligands, and other cytokines

Mammary epithelial cells are exposed to a complex stromal microenvironment containing positive (epidermal growth factor [EGF], insulin-like growth factor [IGF]) or negative growth factors and cytokines (transforming growth factor [TGF] β , leukemia inhibitory factor [LIF], death ligands). The ratio between these

Table 23-1. Transcription profiles of survival and death-related genes upregulated during the first 4 days of involution in the mouse mammary tissue

Transcription profiles	Inv1	Inv2	Inv3	Inv4
Survival genes		<i>Nfkb2</i>		<i>Bcl-x</i> <i>Mcl1</i>
Death genes	<i>Lifr</i> <i>FasL (Tnfsf6)</i> <i>Trail (Tnfsf10)</i> <i>Tweak (Tnfsf12)</i> <i>Tnf (Tnfa)</i> p53	<i>Casp4 (Casp11)</i> <i>Casp12</i> p21	<i>Bax</i> <i>Casp7</i> <i>Fas (Tnfrsf6)</i> <i>Tnfrsf1a</i> <i>Fadd</i> <i>Stat3</i> <i>Tgfb3</i>	<i>Igfbp5</i> <i>Casp1</i> <i>Apaf1</i> <i>Tgfb1</i>

Note: Summary of microarray data (Clarkson et al. 2003 and 2004, Stein et al. 2004 and 2007). Four patterns of gene transcription are observed during involution (Inv1, Inv2, Inv3, Inv4). The dotted lines on the gene expression profiles highlight day 10 of lactation. The following time points correspond to 12-, 24-, 48-, 72-, and 96-hour involution. Inv1 corresponds to a rapid but transient upregulation 12 hours after weaning. Inv2 profiles show a peak at 12 hours, followed by a slow decrease in expression. Inv3 patterns exhibit a gene upregulation by 24/48 hours with prolonged expression. Inv4 corresponds to a delayed and progressive increase in transcription up to at least 4 days. Of note, the survival gene *Akt1* is downregulated during the first 4 days of involution.

mitogen/survival and apoptotic factors regulates the epithelial cell fate through autocrine and paracrine pathways.

2.2.2. Death ligands and death receptor pathway

Microarray analyses of transcription during early involution indicate a rapid and transient increase in the mRNAs of several members of the tumor necrosis factor (TNF) superfamily of death ligands (*Tnf*, *Trail*, *FasL*, and *tweak*) and their receptors (*Tnfr1*, *Fas*, *Dr4*). Increased nuclear factor kappa B (NF- κ B) activity correlates with the rapid activation of these death ligands, suggesting that NF- κ B could be the transcription factor for these death genes.

FAS protein is present in the mammary epithelium during normal breast development, absent during pregnancy and lactation, and returns after weaning. On the other hand, FAS-L protein is present during pregnancy, lactation, and weaning, but not in the virgin mouse. The overlapping expression of FAS and FAS-L during involution matches the occurrence of apoptosis in the mammary epithelium. Lack of FAS or FAS-L expression in transgenic mice prevents apoptosis of mammary epithe-

lial cells during the first 3 days of involution, suggesting that the FAS/FAS-L system may play an important role in early stages of involution.

These results demonstrate that autocrine death receptor signaling contributes to early apoptosis induction during mammary involution. Several death ligands act in concert during this physiologic process. However, they are not the exclusive death mediators because their absence only delays involution rather than preventing it. Regulators of the intrinsic pathway are upregulated at a later time point of the first involution stage, indicating a progressive shift in the cell death machinery from the extrinsic to the intrinsic pathway.

2.2.3. TGF β 3 proapoptotic pathway

TGF β 1, 2, and 3 are multifunctional cytokines that play critical roles in every phase of the mammary gland development. They are expressed by mammary epithelial cells as inactive precursors binding to the extracellular matrix and later activated by proteolytic cleavage. Among the three TGF β s, TGF β 3 seems to be the primary isoform regulating apoptosis in the mammary epithelium.

At weaning, TGF β 3 mRNA and protein are dramatically upregulated in the mouse alveolar and ductal epithelium, and this expression precedes the onset of apoptosis in the first phase of involution. Transplantation of neonatal mammary tissue from *Tgf β 3* null mice into recipient hosts results in normal development and lactation but causes reduced apoptosis upon milk stasis compared with wild-type controls. Hormonal reconstitution and inhibition of suckling by teat sealing showed that TGF β 3 is induced locally by milk stasis and not by the levels of circulating hormones.

TGF β s signal through an hetero-tetrameric receptor complex composed of type I and type II TGF serine threonine kinase receptors (TGF β RI and TGF β RII), leading to the phosphorylation of SMADs. In the involuting mammary gland, TGF β 3 induces apoptosis through two classical signaling mediators: the transcription factors SMAD3 and SMAD4. Indeed, like in *Tgf β 3* null mice, the mammary gland of *Smad3* null transplants undergoes reduced alveolar apoptosis upon forced weaning. Moreover, directed expression of TGF β 3 in the alveolar epithelium of lactating mice leads to apoptosis of those cells with the concomitant nuclear localization of SMAD4. Mammary epithelial cells over-expressing TGF β 3 also show strong nuclear localization of phosphorylated STAT3 during early involution, suggesting that STAT3 may be a downstream target of TGF β 3 signaling.

According to several studies, TGF β signaling contributes to the negative regulation of the downstream survival kinase AKT. Bailey and coworkers reported that porcine TGF β inhibits AKT activity and triggers apoptosis of mouse mammary epithelial cells in culture and that prolactin prevents this TGF β -induced apoptosis. The same authors also show that over-expression of a dominant-negative TGF β type II receptor in the mouse mammary epithelium causes a hyperplastic prolactin-dependent alveolar development and a delayed involution with increased phosphorylated AKT levels. These results suggest that prolactin and TGF β s exert opposing effects on alveolar development through careful regulation of apoptosis by AKT.

Taken together, literature data provide evidence that TGF β 3 is one of the local autocrine factors that induces apoptosis of the mammary epithelium during the first phase of involution, possibly through downstream regulation of survival AKT kinase.

2.2.4. LIF-STAT3 proapoptotic signaling

STATs are transcription factors activated by phosphorylation that mediate signaling from cytokines and

growth factors. It is now known that LIF is the major paracrine cytokine that activates STAT3 in vivo in early involution. LIF receptor is upregulated at the onset of involution. Moreover, knockout of LIF in mice suppresses the phosphorylation of STAT3 after weaning and delays involution. TGF β 3 has also been identified as an additional upstream regulator of STAT3 activity during involution.

STAT3 is activated by phosphorylation at the onset of involution and is critical for the first apoptotic phase of this process. *Stat3* null mammary glands indeed show a reduced epithelial apoptosis and a dramatic delay in involution upon forced weaning.

STAT3 regulates the transcription of a number of genes promoting apoptosis in mammary epithelial cells. Many of these genes are upregulated at the transition from lactation to involution. A number of targets have been identified in vitro in mammary epithelial cells. STAT3 targets include CCAAT/enhancer binding protein delta (*C/EBP δ*), suppressor of cytokine signaling 3 (*Socs 3*), *c-fos*, *Smad1*, B-cell leukemia lymphoma 3 (*Bcl3*), and the regulatory subunits p55 α /50 α of phosphoinositide 3-kinase (PI3K).

Stat3 and *Lif* null mammary epitheliums express reduced levels of C/EBP δ , confirming C/EBP δ as a STAT3 target in vivo. C/EBP δ is a critical regulator of the proapoptotic genes *Bak*, *Igfbp-5*, *p53*, and *Sgp2* during mammary gland involution. Moreover, *C/EBP δ* disruption in the mouse epithelium delays the onset of involution and the expression of proapoptotic factors such as *p53* and the IGF binding protein IGFBP-5.

Other important targets of STAT3 are the PI3K regulatory subunits. Their induction mediates a negative switch in PI3K survival signaling and a decrease in downstream AKT1 activation.

Overall, the LIF-STAT3 pathway emerges as a key regulator of apoptosis within the mammary gland, stimulating the expression of proapoptotic genes such as *p53* or *Bak* while inhibiting two major survival pathways, the IGF and PI3K/AKT pathways.

2.2.5. IGF survival signaling

The IGF system is composed of three ligands (IGF-1, IGF-2, insulin), three receptors (IGF-1R, IGF-2R, and IR), and six IGF binding proteins (IGFBPs). IGFs are both systemic growth factors coming from the liver and autocrine/paracrine factors secreted locally by many tissues, including breast. They act as cellular mitogens and survival factors, protecting epithelial cells from apoptosis in a wide variety of conditions. IGF-1 and 2 exert their physiologic effects mostly through the binding

to the tyrosine kinase receptor IGF-1R. Ligand stimulation triggers the receptor autophosphorylation and the subsequent recruitment of adaptor proteins (IRS-1, SHC, 14-3-3). Phosphorylation of IRS-1 activates the PI3K/AKT survival pathway, which in turn phosphorylates BAD. The IGF-1R suppresses apoptosis primarily through the PI3K pathway, but two alternative routes, the mitogen-activated protein kinase (MAPK) pathway and the translocation of RAF to the mitochondria, also contribute to BAD phosphorylation. The bioavailability and function of IGFs are modulated by high-affinity associations with IGFBPs. IGFBPs sequester the IGFs and prevent them from binding to their receptors.

Components of the IGF system are differentially expressed in the developing mammary gland. IGF-1R is expressed only in the epithelium, whereas IGFs and IGFBPs are synthesized by stromal cells (i.e., fibroblasts and adipocytes) or epithelial cells, depending on the particular isoform and developmental stage. These data emphasize the importance of stromal-epithelial interactions for the regulation of IGF signaling in the mammary gland.

Substantial *in vitro* and *in vivo* evidence supports a role for IGF-1 in the regulation of mammary epithelial cell survival. Inhibition of IGF signaling triggers apoptosis, whereas extra IGFs suppress it. *In vitro*, exogenous IGF-1 suppresses apoptosis of primary mammary epithelial cells in culture by inducing AKT activation and FKHRL1 phosphorylation. IGF-1R over-expression induces proliferation and antiapoptotic signaling in a three-dimensional culture model of breast epithelial cells. Mammary gland models confirm the importance of IGFs as survival factors *in vivo*. Over-expression of IGF-1 or IGF-2 in the mammary gland of transgenic mice results in reduced apoptosis and incomplete involution. Sustained activation of AKT is also detected in IGF-2 transgenic mice.

The survival function of IGFs are counteracted by IGFBP-5 during involution. Indeed, Marshman et al. report that IGFBP-5 protein is upregulated during the early apoptotic phase of involution and that IGFBP-5 is able to inhibit IGF-mediated survival signaling to cause apoptosis of primary mammary epithelial cells in culture. Loss-of-function and gain-of-function studies in mice confirm the proapoptotic role of IGFBP-5 during involution. Knockout of *Igfbp-5* in the mouse mammary epithelium does not affect the mammary gland development but reduces apoptosis in early involution. On the other hand, directed expression of IGFBP-5 in the mammary gland of transgenic mice

induces premature cell death and impaired mammary development, together with increased expression of the proapoptotic molecule caspase-3 and decreased expression of prosurvival members of the BCL-2 family.

Current data thus support a role for the IGFs in apoptosis regulation in the mammary gland and highlight IGFBP-5 as a physiologic inhibitor of IGF survival signaling in this organ.

2.2.6. Regulation by adhesion

Mammary epithelial cells, like all normal adherent cells, are dependent on anchorage for survival. They are anchored to the ECM and to neighboring epithelial cells via transmembrane adhesion proteins such as integrins and cadherins. Integrins are well-known adhesion receptors existing as α - β heterodimers and linking the ECM with the cell interior (cytoskeleton and intracellular signaling molecules). The alpha subunit interacts with specific ligands from the ECM, whereas the beta subunit recruits intracellular signaling molecules such as focal adhesion kinase (FAK) or integrin-linked kinase (ILK). Integrins, which regulate cell architecture, are also known to cooperate with growth factor receptors to control cell survival. Anchorage survival signal is transduced intracellularly by β 1 integrins to FAK and ILK, which in turn activate the survival kinases PI3K and AKT, respectively. Cadherins are the major cell-cell adhesion molecules of adherens junctions and desmosomes that bind to identical partners in neighboring cells. They are linked intracellularly to the actin cytoskeleton via β -catenins and α -catenins. Cytoplasmic β -catenin is degraded by the ubiquitin-proteasome pathway. When stabilized by WNT and non-WNT pathways, cytoplasmic β -catenins can translocate to the nucleus, where they act as transcription factors to mediate proliferation and survival signals.

In nontransformed epithelial cells, disruption of cell-matrix and cell-cell adhesion leads to detachment-induced apoptosis, called anoikis. Artificial disruption of cell-matrix interactions by anti- β 1 integrin antibodies or over-expression of the matrix metalloproteinase 3 (MMP-3) induces anoikis in mammary epithelial cells in culture. Conditional deletion of E-cadherin or α -catenin in mouse mammary glands causes dramatic apoptosis at parturition, which generates a mutant mammary gland with involution-like features. In physiologic conditions, shedding of epithelial cells into the ductal lumen is detected during ductal morphogenesis and as early as 12 hours after forced weaning during involution.

This effect precedes caspase-3 activation and involves the disruption of both cadherin and integrin signaling. Indeed, truncation of the β -catenin-binding domain of E-cadherin was shown to precede epithelial apoptosis in early involution. Moreover, sFRP4, a decoy receptor for WNT ligands, is upregulated at involution and hence inhibits WNT signaling, stabilizing β -catenin. Immunohistochemistry studies revealed that β 1 integrin adopts an inactive conformation or is downregulated together with FAK in the first reversible phase of involution. MMPs that degrade the ECM are maintained in the inactive state by tissue inhibitors of MMPs in early involution, and their activities are then upregulated to enable matrix degradation and remodeling in the second involution phase. There is evidence for the degradation of ECM components laminin and fibronectin concomitantly with increased MMP activities during involution.

The adhesion system thus exerts a stringent control on epithelial cell fate by sensing the microenvironmental context. Proper adhesion activates survival signals that synergize with growth factor and cytokine signaling. Loss of integrin and cadherin adhesion and signaling has been involved in anoikis during both phases of the involution process. First, milk stasis causes an alveolar stretch, which probably alters cell adhesion and thereby triggers a first wave of anoikis. Then, upregulation of MMP activities in the second phase results in the degradation of the ECM and subsequent anoikis of remaining secretory alveoli.

2.2.7. PI3K/AKT pathway: molecular hub for survival signals

The PI3K/AKT pathway is known as a critical survival pathway in cells. PI3K activates the kinase AKT, which in turn phosphorylates and inactivates proapoptotic factors such as caspase-9, BAD, or FKHRL1.

In the mammary gland, levels of *Akt* mRNA increase slightly during pregnancy and more dramatically during lactation, dropping sharply as the gland begins to involute. The activated AKT protein levels are likewise decreased during the first stage of involution.

Shutting down this survival pathway is important for the course of involution, as demonstrated in vivo. Tissue-specific expression of AKT1 has been shown to delay involution in transgenic mice. By contrast, expression of phosphatase and tensin homolog (PTEN), a negative regulator of PI3K, enhances apoptosis in the mouse mammary gland and conversely, conditional deletion of this gene delays involution.

As previously described in this chapter, multiple cell surface stimuli feed into the PI3K/AKT pathway within the mammary epithelium. These include:

- Prolactin signals through JAK2 to FYN and CBL, which in turn activates the PI3K/AKT pathway. This pathway is downregulated during involution.
- IGF signaling activates PI3K and is suppressed by IGFBP-5 during early involution.
- Interaction of epithelial cell surface integrins with the extracellular matrix proteins provides additional survival signals to PI3K/AKT through FAK or ILK.
- PI3K negative regulatory subunits are upregulated by STAT3 during involution and mediate a negative switch in PI3K signaling, facilitating the decrease of active AKT.

Loss of prolactin, sequestration of IGFs by IGFBPs, and disruption of epithelium-matrix interactions all reduce signaling to PI3K/AKT in the involuting breast. Moreover, STAT3 upregulates the expression of PI3K regulatory subunits, thereby mediating a negative switch in PI3K signaling. Overall, AKT1 emerges as the critical molecular nexus for survival/death signals in the mammary epithelium.

2.2.8. Downstream regulators of apoptosis: the BCL-2 family members

BCL-2 family members are important downstream regulators of the mitochondrial apoptotic pathway that can act either as death promoters (BIM, BAX, BAD, BAK, BCL-XS) or death inhibitors (BCL-2, BCL-W, BCL-XL). The relative ratios of these various pro- and antiapoptotic members determine the sensitivity or resistance of the cells to diverse apoptotic stimuli.

The mammary epithelium expresses a number of BCL-2 family members, including BIM, BAX, BAK, BAD, BCL-X, BCL-W, BFL-1, MCL-1, and BCL-2. Expression patterns of these BCL-2 proteins vary throughout mammary gland development. For instance, BCL-2 expression is dependent on estrogen and fluctuates with the cyclic changes of estrogen levels during the menstrual cycle. BCL-2 protein is expressed throughout pregnancy and lactation and drops at involution.

The functional roles of individual BCL-2 family members have been investigated using dominant gain-of-function and loss-of-function in mice (germline or tissue-specific loss of function). BIM, BAX, BCL-2, and BCL-X are expressed at the TEBs during pubertal development. Disruption of *Bim* in mice prevents the induction of apoptosis and clearing of the lumen in

TEBs during puberty, thereby demonstrating the importance of Bim for ductal morphogenesis. By contrast, no major involution defects are observed in *Bim* null mammary glands after forced weaning, suggesting that the apoptotic involution mechanism is distinct from luminal clearing during development. Over-expression of BCL-2 was found to partially suppress body cell apoptosis and to disrupt TEB structure. However, lumen formation was not inhibited, and mature virgin mammary glands developed normally in this transgenic model. In mature females, antiapoptotic BCL-2 and BCL-W mRNA and protein are downregulated during involution, whereas proapoptotic BAX, BAK, and BAD proteins are upregulated during lactation and early involution. The relative levels of *Bcl-xS/Bcl-xL* mRNAs also increase at the onset of involution. Studies in transgenic mice demonstrate that several BCL-2 family members contribute to the molecular control of apoptosis in the involuting breast. Over-expression of BCL-2 in *wap-Bcl-2* transgenic mice inhibits alveolar cell apoptosis during involution. Conditional deletion of *Bcl-x* in the mouse mammary epithelium results in accelerated apoptosis during involution but does not compromise mammary function during lactation. Last but not least, disruption of *Bax* in the mammary epithelium reduces apoptosis levels during the first stage of involution but does not affect the second phase of involution.

p53, a well-known transcription factor that regulates the expression of several BCL-2 family members (*Bax*, *Bak*, *Noxa*, *Puma*, *Bcl-2*), is upregulated at the onset of involution. Its disruption delays involution, highlighting a physiologic role in this process. Investigators wondered whether p53 could be involved in *Bax* upregulation in the involuting breast. However, if p53 does regulate the transcription of the cell cycle inhibitor p21, it does not seem to induce *Bax*. The role of upregulated p53 in inducing proapoptotic *Noxa* and *Puma* or in downregulating *Bcl-2* has not been investigated.

Altogether, the current literature shows that BIM is required for lumen clearance during ductal morphogenesis, whereas BCL-2, BCL-X, and BAX are important for the molecular control of apoptosis during involution.

In summary, apoptosis in the normal breast is under the molecular control of several extracellular and intracellular factors. The BCL-2 family member BIM is required for lumen clearance during epithelial morphogenesis. Involution is the result of the coordinated regulation of a complex network of proteins (Figure 23-2). Milk accumulation causes an alveolar stretch, leading to the disruption of prosurvival cell-matrix and cell-cell

adhesions. Truncation of the β -catenin binding domain of E-cadherin was shown to precede epithelial apoptosis in early mammary involution. Local milk stasis also induces the expression of several proapoptotic cytokines – LIF, TGF β 3, and death ligands – that trigger apoptosis through the death receptor pathway and the STAT3 pathway. Survival pathways such as IGF and the PI3K/AKT signaling pathway are inhibited, whereas downstream targets are upregulated to ensure the transition to the second phase. For instance, dimeric transcription factors AP-1 and macrophage markers are upregulated at the end of the first phase to initiate the shift to the second phase of involution. AP-1 is known to regulate the transcription of the matrix metalloproteinase stromelysin-1 (MMP-3), whereas macrophage markers help recruit macrophages for the phagocytosis of apoptotic bodies. In the second phase of involution, activated matrix metalloproteinases degrade the ECM and finally trigger the massive anoikis of the remaining secretory alveoli.

3. APOPTOSIS IN BREAST CANCER

Disruption of balance between cell death and proliferation is considered a major factor in the growth of tumors or their regression during therapy. This balance can be disrupted in two ways in tumors: by increasing proliferation and/or decreasing apoptosis. There is evidence that tumor growth results from both uncontrolled proliferation and reduced apoptosis. In premalignant stages, major alterations in apoptosis, cell proliferation, and cell cycle regulators would arise, allowing the later progression of the disease.

The susceptibility of the mammary gland to tumorigenesis is influenced by its development particularly during puberty and pregnancy, when marked changes in cell proliferation, invasion, differentiation, and apoptosis occur. Indeed, terminal ducts that are highly proliferative in early adulthood are all the more susceptible to carcinogen exposure at that period. Moreover, the process of involution co-opts some of the programs of wound healing, creating a proinflammatory stroma that can promote tumor progression and that explains the high rate of metastases reported in pregnancy-associated breast cancer. In fact, the developing breast shares many properties (proliferation, invasion, angiogenesis, proinflammatory stroma) with breast cancer, and many signaling pathways that regulate processes such as invasion, proliferation, or apoptosis in the normal breast can be corrupted by tumor cells to their own advantage.

The extraordinary developing capacity of the normal breast underlies its great susceptibility to tumorigenesis and may explain why breast cancer is the most common type of nonskin cancer and the second leading cause of cancer death in American women. Most breast cancers arise from the epithelium in the undifferentiated terminal duct lobular unit, leading to cancer of the ducts (ductal carcinoma, approximately 90% of breast carcinomas) or cancer in the milk-producing glands (lobular carcinoma, approximately 10% of breast carcinomas). The development of breast cancer has been described as a multistep process with progressive phenotypic changes from hyperplasia with or without atypia through in situ carcinoma to invasive carcinoma capable of invading surrounding tissues and eventually metastasizing. The role of apoptosis in breast carcinogenesis and progression has been the focus of many investigations, and the main results are discussed next.

3.1. Apoptosis in breast tumorigenesis and cancer progression

Apoptosis status (occurrence, apoptotic rates, molecular regulation) has been analyzed at different stages of breast cancer development, in 3D culture systems, murine models, and patient samples. The number of apoptotic cells as a percentage of cells present, or the number of apoptotic cells per square millimeter of neoplastic tissue, is usually described as the apoptotic index (AI), as opposed to the mitotic index (MI), the percentage of proliferating cells.

In contrast with normal breast, premalignant breast cancer lesions fail to respond to normal apoptotic stimuli for lumen clearance and mammary involution. Indeed, hyperplasia with atypia and carcinoma in situ are characterized by a complete or partially filled lumen. Debnath et al. used a 3D culture of the mammary epithelial cell line MCF-10A to investigate the importance of enhanced proliferation versus apoptosis inhibition for lumen filling. Neither enhancing proliferation (by over-expressing mitogenic oncoproteins) or inhibiting apoptosis (by over-expressing BCL-2 or BCL-XL) was sufficient to induce lumen filling. By contrast, oncoproteins such as ERBB2 and IGF-1R that simultaneously promote proliferation and prevent apoptosis induced lumen filling. ERBB2 was shown to prevent normal luminal apoptosis by downregulating BIM. Therefore, in 3D culture models, enhanced proliferation requires a concomitantly blocked apoptosis to cause neoplasia. Consistently, hyperplasia implants in mice are also unresponsive to normal apoptotic signals during mammary gland involution and fail to regress upon

forced weaning. Phosphorylated AKT1 and BCL-2 protein levels are higher in those hyperplasias than in the normal regressed mammary gland, suggesting that inhibition of cell death creates a permissive cellular environment for neoplastic transformation. This inhibition of apoptosis is consistent with reduced AI in a carcinogenesis model in rats. In this animal model, mammary tumors are preceded by hyperplastic and premalignant lesions arising mostly in TEBs, as well as in ducts and alveoli. Quantification of MI and AI showed that the percentage of proliferating cells is similar in TEBs to those in terminal end bud hyperplasia (TEBH), carcinomas in situ (CIS), and carcinomas, whereas the percentage of apoptotic cells (AI) is relatively high in TEBs and decreased in TEBH, CIS, and carcinomas. This indicates that, in this model, neoplastic transformation of mammary epithelial cells in TEBs is not associated with an increase in cell proliferation, but rather with a decrease in apoptotic cell death. In patients, reduced apoptosis is also detected in noninvolved tissue from cancer-containing breasts when compared with age-matched benign tumors and normal breast tissue from women without cancer after menopause. Hyperplasias are thus associated with reduced apoptosis when compared with normal tissue both in mouse models and in the normal surrounding tissue of breast tumors. The reduction in apoptosis may lead to the preservation of genetically aberrant cells, hence favoring neoplastic development.

Whereas hyperplasia formation requires reduced apoptosis, malignant progression from hyperplasia to invasive carcinoma is usually associated with an increase in both cell proliferation and apoptosis. In breast cancer, high AIs have been correlated with several pathologic parameters, such as high MI, high tumor grade, lack of tubule formation, tumor necrosis, absence of BCL-2 and estrogen receptor (ER) expression, expression of p53, and poor overall survival. Rates of apoptosis are thus related to tumor grade and are higher in more aggressive tumors that exhibit higher rates of proliferation. The current hypothesis is that apoptosis may help selecting clonal subpopulations with high growth potential during breast cancer progression.

Discordant results are found for the transition from in situ carcinoma to invasive carcinoma. Some investigators reported a reduction in AIs from ductal carcinomas in situ to invasive carcinomas. These discrepancies could come from the different methods used for apoptosis detection (microscope counting or terminal deoxynucleotidyl transferase dUTP nick end labeling [TUNEL]) or from heterogeneous sample cohorts (age and number of patients, tissue differentiation, etc.). Mommers et al.

proposed two progression models according to the cell differentiation status. In their study, the progression from ductal carcinoma in situ (DCIS) to invasive carcinoma (IC) was related to an increase of MI with no significant change in AI in well-differentiated lesions and to an increase of MIs and a two-fold decrease in AIs in poorly differentiated tumors. Yamamoto et al. found that advanced breast cancer with distant metastases negative for p73 have low AI and high MI, suggesting an inhibition of apoptosis in those tumors. After analysis of 91 invasive breast carcinomas, Wu and coworkers concluded that low AIs are related to axillary lymph node metastasis and shorter overall survival. Along these lines, transgenic mouse tumor models suggest that in proliferative lesions with high rates of proliferation and apoptosis, progression to frankly malignant aggressive invasive tumors occurs if apoptosis is impaired and proliferation maintained. Therefore, it is likely that the most aggressive metastatic tumors acquire further genetic and epigenetic defects enabling evasion from apoptosis while they invade foreign tissues.

Apoptosis contributes to spontaneous cell death in tumors, but also to cell death induced by various anticancer agents (hormonal therapy, chemotherapy, radiotherapy, immunotherapy). Measurable increases of apoptosis and decreases in proliferation are detected within 24 hours of the start of effective treatments. When those treatments fail, dysregulation of apoptotic pathways may be a cause.

3.2. Molecular dysregulation of apoptosis in breast cancer

3.2.1. Altered expression of death ligands and their receptors in breast cancer

Faulty regulation of the FAS and TNF-related apoptosis-inducing ligand (TRAIL) system has been described in a variety of human tumors, including breast carcinomas. Breast carcinomas display an altered expression of death ligands FAS-L and TRAIL and their respective receptors. They generally express much more FAS-L protein and less FAS receptor than normal breast tissue or benign tumors. The differentially expressed FAS-L and FAS are inversely correlated with node status and tumor size. A ratio of *Fas-L* to *Fas* mRNA greater than 1 was found to be significantly associated with higher tumor grade, shorter disease-free survival, and increased mortality. Increased expression of FAS-L compared with its receptor would enable breast cancer cells to bypass immune surveillance by inducing apoptosis in FAS-positive infiltrating lymphocytes, thereby facilitating tissue invasion.

In contrast with the FAS/FAS-L system, TRAIL is strongly expressed in 30% to 50% of breast cancers, but the associated death receptors DR4 and DR5 are not downregulated. An immunohistochemistry study of 90 breast cancer patients with invasive ductal carcinoma identified DR4 as the prominent TRAIL receptor expressed and correlated its expression with tumor grade in invasive ductal carcinoma. In the same study, ERBB2-positive tumors were found to express higher levels of both DR5 and TRAIL than ERBB2-negative tissues. The survival of breast tumors that concomitantly express TRAIL and its receptors may be due to altered signal transduction downstream of the death receptors or inactivating mutations in the death receptor itself. Using gene silencing, Day et al. showed that the caspase 8 inhibitor cellular FLICE-like inhibitory protein (c-FLIP) was required for MCF-7 breast cancer cells growth and survival both in vitro and in vivo within tumor xenografts. Inactivating mutations in death receptors have also been found in metastatic breast cancers. TRAIL expression is downregulated by anchorage in breast cancer cells, suggesting that TRAIL-dependent apoptosis may play a role in anoikis of breast epithelial cells. Inactivating mutations in death receptors would therefore contribute to inhibit TRAIL-dependent anoikis of metastatic breast cancer cells.

3.2.2. Deregulation of prosurvival growth factors and their receptors

Growth factors and their receptors function in a complex and interconnected network critical for both the development of the normal breast and the pathogenesis and progression of breast cancer. Their deregulation in cancer leads to the hyperactivation of survival signaling pathways and subsequent evasion from apoptosis.

The EGF receptor (EGFR) family consists of four related receptor tyrosine kinases (EGFR, ERBB2/HER2, ERBB3/HER3, and ERBB4/HER4). HER2 is the preferred heterodimerization partner of all ERBB receptors and can mediate signal transduction of all ERBB members when they bind to their cognate ligands such as EGF, TGF- α , amphiregulin, or neuregulins. Over-expression of EGFR and ERBB2, which causes growth factor independence, is frequent in breast cancer and is linked with a more aggressive course of the disease. Over-expression of ERBB receptors leads to the enhanced activation of two main survival pathways: the MAPK and PI3K/AKT pathways. Hyperactivation of survival signaling has been shown to confer resistance to apoptosis induced by hormonal or chemotherapy, whereas inhibition of this downstream signaling by trastuzumab, a monoclonal

antibody against HER2, effectively induces apoptosis in HER2-over-expressing metastatic breast cancer.

The IGF/IGF-1R system exerts an antiapoptotic function in both the normal and neoplastic breast. Among all growth factor receptors, IGF-1R displays one of the most potent antiapoptotic activities because it protects cells from apoptosis via multiple signaling pathways, all leading to the phosphorylation of BAD.

A disrupted balance in the IGF system can cause excessive survival signals as observed in breast tumors. A change in IGF expression has been found in breast carcinomas. High endocrine circulating levels of IGF-1 have been associated with an increased risk of breast cancer in premenopausal women. Moreover, stromal cells surrounding the normal breast epithelium secrete IGF-1, whereas those surrounding the malignant epithelium secrete IGF-2, suggesting that transformation of breast cells may be associated with a switch from stromal IGF-1 to IGF-2 expression. IGF-1R is over-expressed and/or constitutively activated in breast cancer tissue compared with normal or benign tumoral tissue. This over-expression protects breast cancer cells from apoptosis and enhances their survival *in vitro* and *in vivo*, whereas downregulation or functional inactivation of IGF-1R causes massive apoptosis in tumor xenografts. Inactivating mutations of tumor suppressors and cross-talk with hormone and growth factor receptors signaling contribute to the regulation of IGF-1R expression and activity in breast neoplasia. Indeed, expression of IGF-1R is inhibited by tumor suppressors such as wild-type p53 and breast cancer 1 (BRCA-1) but upregulated by mutant p53. The expression and activity of IGFR is also regulated by steroid hormone receptors (SR) and EGF receptors via a cross-talk in signaling networks. Growth factors such as EGF and IGF are known to influence the expression and activity of SRs. In turn, the expression of growth factor receptors, their ligands, and signaling molecules is often controlled by SRs. For instance, estrogens induce expression of IGFs, IGF-1R, and IRS-1 and IRS-2, whereas IGF-1 enhances the expression and activation of ER in breast cancer cells. EGF also stimulates IGF-1R expression, and its receptor EGFR can interact with IGF-1R and regulate its stability. IGF-1R/HER2 heterodimerization was shown to contribute to trastuzumab resistance of breast cancer cells. Thus both the IGF ligand and receptor play a major role in tumor cell survival and resistance to apoptosis induced by anticancer therapies.

TGF β s are well-known growth inhibitory and proapoptotic factors contributing to normal mammary development. In breast cancer, TGF β s play a dual role in mammary tumorigenesis, acting as a tumor suppressor in early stages of cancer and promoting invasion and metastasis at later stages. One proposed mechanism

to explain TGF β tumor suppressor function involves the TGF β 1-dependent repression of human telomerase reverse transcriptase causing cellular senescence in the mammary stem cell population. Enhanced expression of TGF β and downregulation of TGF β receptor 2 have been associated with breast cancer progression and aggressiveness of the disease. The shift from tumor suppressor to tumor promoter function could be due to mutations in *Tgfb2* acquired during the course of the disease. Indeed, an insertion polymorphism in *Tgfb2* leading to an increased *Tgfb2* promoter activity was associated with lymph node metastases and advanced breast tumor stage independently of estrogen and progesterone receptor status. This study suggests that increased TGF β 2 expression in breast tumors bearing this allele may promote metastasis. According to Yu et al., activation of latent TGF β 2 in CD44-MMP-9 complexes on the surface of mouse mammary carcinoma would be required for tumor cell survival during metastatic colony formation.

Recent studies have identified possible molecular mechanisms by which TGF β promotes survival. Ehata and colleagues report that TGF β promotes survival of mammary carcinoma cells through induction of antiapoptotic transcription factor DEC1. Several groups also demonstrate that activation of the PI3K/AKT is involved. Yi and coworkers showed that type I TGF β receptors could bind and activate PI3K in COS-7 epithelial cells, suggesting that PI3K/AKT signaling can be an effector of the oncogenic function of TGF β . Dumont et al. used MDA-MB-231 breast cancer cells stably expressing a kinase-inactive type II TGF β receptor to show that TGF β promotes motility through mechanisms independent of SMAD signaling, possibly involving the activation of the PI3K/AKT and/or MAPK pathways. A recent study from Wang et al. suggests that TGF β -dependent activation of the PI3K/AKT pathway could be involved in trastuzumab/herceptin resistance. In HER2-over-expressing cancer cells, TGF β indeed synergized with the HER2 signaling network to activate the PI3K/AKT pathway and promote cancer cell survival, migration, and resistance to trastuzumab. Finally, TGF β signaling is regulated by estrogens in ER-positive tumors. Antiestrogens such as tamoxifen can induce TGF β 1 expression and proapoptotic activity in human MCF-7 breast cancer cells *in vitro*.

3.2.3. Alterations in cell adhesion and resistance to anoikis

A hallmark of cancer is anchorage-dependent growth, which allows cancer cells to survive when they pile up and detach from the basement membrane. This

resistance to anoikis is mediated in part by alterations in the adhesion system and its related signaling. Breast cancer progression is associated with a change in integrin and cadherin expression profiles, referred as the integrin and cadherin switches. Invasive breast cancer cells tend to downregulate the integrins such as $\alpha 2\beta 1$ that mediate their adhesion to the basement membrane and to upregulate integrins such as $\alpha v\beta 6$ or $\alpha 6\beta 4$, which promote survival, migration, and invasion during metastasis. In breast cancer cells over-expressing ERBB2, integrins $\alpha 6\beta 4$ colocalize and associate with ERBB2, which potentiates the survival, proliferative, and invasive capacities of those neoplastic cells. A cadherin switch also occurs during breast cancer progression, with downregulation of the invasion suppressor E-cadherin and upregulation of N- and P-cadherins. E-cadherin is considered as an invasion suppressor because loss of E-cadherin cell adhesion leads to anoikis and thus prevents tumor cells from invading surrounding tissues. Reduced E-cadherin expression therefore favors dissemination and in general correlates with noninvasive phenotypes. On the other hand, N- and P-cadherins would facilitate invasion and metastasis by promoting tumor cell affinity for the stromal and endothelial cells of distant sites. Regain of E-cadherin expression is sometimes observed in some metastases to favor survival and reattachment of metastases.

β -catenin is often upregulated and stabilized in breast cancer, thereby providing additional survival cues. MMPs are also commonly expressed and secreted at high levels by stromal cells (fibroblasts and tumor-infiltrating macrophages) in invasive breast cancer. They are known to stimulate proliferation, activation of growth factors and their receptors, and resistance to apoptosis. By degrading the ECM of primary neoplastic cells, MMPs would favor the anoikis of a majority of epithelial cells but also help to select anoikis-resistant clones. Moreover, several MMPs promote tumor cell proliferation and survival by releasing growth factors and death ligand FAS-L bound to ECM components.

Overall, dysregulated cell adhesion is one of the features acquired by breast tumor cells to bypass anoikis and which allows them to survive in an unpolarized state in foreign microenvironments.

3.2.4. Enhanced activation of the PI3K/AKT pathway in breast cancer

The PI3K/AKT pathway is frequently activated in breast cancer through diverse mechanisms, including membrane receptor signaling and/or mutations in PI3K or its negative regulator PTEN. PI3K/AKT is activated by upstream membrane receptor pathways (i.e., ER, ERBB2,

EGFR, integrin receptor signaling) that are often dysregulated in cancer. Activating mutations in PI3K are common in invasive ductal carcinomas of the breast and are associated with poor prognosis. Moreover, inactivating mutations in the tumor suppressor PTEN occur in up to 50% of breast cancer.

Zhou et al. showed that phosphorylation of AKT increases progressively during breast cancer progression from normal epithelium to hyperplasia and invasive carcinoma. AKT activity increases as breast cancer malignancy intensifies, resulting in a poor prognosis. Several reports indicate a positive correlation between active AKT, over-expression of ERBB2, and histological grade of the tumor. AKT activation has also been involved in breast cancer cells' resistance to many anticancer therapies; thus inhibition of the PI3K/AKT pathway is being investigated as a new therapeutic strategy for breast cancer patients.

Therefore, activation of the survival kinase AKT significantly contributes to the progression of breast cancer and resistance to radio- and chemotherapy.

3.2.5. p53 inactivation in breast cancer

Wild-type p53 is a tumor suppressor that plays a central role in maintaining cellular genetic integrity by preventing DNA-damaged cells from further proliferation. Inactivation of p53 is a major event in tumorigenesis.

Mutation and deletion of p53 are the most common genetic defects seen in clinical cancer. Somatic mutations of p53 are found with high frequency in both the epithelium and stroma of invasive breast carcinomas. p53 mutations generally result in impaired transcriptional activity and increased protein stability. Large case studies demonstrated that p53 mutations are independent markers of poor prognosis in breast cancer and that the exact type and position of the mutation influences disease outcome. Accumulation of stabilized p53 can be detected in early breast lesions, and its occurrence increases with tumor progression.

Many tumors with wild-type p53 do not have normal p53 function, suggesting that some oncogenic signals suppress the function of p53. Zhou et al. showed that HER2/neu-mediated resistance to DNA-damaging agents requires the activation of AKT, which enhances MDM2-mediated ubiquitination and degradation of p53. In a recent study, Danes et al. demonstrated that 14-3-3 zeta over-expression is a critical event in early breast disease conferring resistance to anoikis via the downregulation of p53 expression. Mechanistically, 14-3-3 zeta induced hyperactivation of the PI3K/AKT pathway, which led to phosphorylation

and translocation of the MDM2 E3 ligase, resulting in increased p53 degradation. Ectopic expression of p53 restored luminal apoptosis in 14–3–3 zeta-over-expressing MCF10A acini in 3D cultures. The authors concluded that downregulation of p53 is one of the mechanisms by which 14–3–3 zeta alters mammary epithelial cells acini structure and increases the risk of breast cancer. Recent publications also suggest that loss of p53 permits expansion of cancer stem cells in mouse mammary tumors and in human breast cell lines.

Cell and animal experimental studies have linked p53 status with response to therapy. They support a role for wild-type p53 in drug sensitivity and a role for mutant p53 in chemoresistance, but the predictive value of p53 in response to chemotherapy remains unclear in patient studies in which treatment, tumor, and mutation types are often heterogeneous.

3.2.6. Altered expression of BCL-2 family of proteins in breast cancer

Dysregulation of apoptosis contributes to the pathogenesis of breast cancer, at least in part, through an imbalance in BCL-2 family members between antiapoptosis (such as BCL-2/BCL-X) and apoptosis-promoting proteins (BAX).

BCL-2, BCL-X, MCL-1, BAX, BAK, and BAG-1 are expressed in human breast cancers. BAX α , a splice variant of BAX, is expressed in normal breast tissue but only weakly in breast tumors. BCL-2 expression is higher in normal breast tissue from a cancer-containing breast in comparison with controls, suggesting that an increase in BCL-2 antiapoptotic protein favors tumorigenesis. BCL-2 expression is more common in tumors that express ER, whereas BCL-XL is more commonly found among HER2-positive tumors. Bcl-2 associated athanogene-1 (BAG-1) is an antiapoptotic protein that binds to and enhances the antiapoptotic activity of BCL-2. It was shown to modulate the interactions of HSP70 chaperones with other proteins, thereby enhancing their biological activity. BAG-1 interacts with several prosurvival proteins important to tumorigenesis (i.e., BCL-2, RAF-1, steroid hormone receptors, some tyrosine kinase receptors, hepatocyte growth factor receptor, and platelet-derived growth factor receptor). BAG-1 expression is high in the majority of invasive breast carcinomas and is correlated with BCL-2 expression and SR positivity.

Alterations in the relative expression levels of individual BCL-2 family members appear to influence breast cancer progression. Over-expression of BCL-X protein in primary breast cancer is associated with high tumor grade and nodal metastases, suggesting that upregula-

tion of BCL-X protein may be a marker of tumor progression. BCL-XL protein would promote survival of cells in metastatic foci by counteracting the proapoptotic signals in the microenvironment. According to Sierra and colleagues, BCL-XL indeed mediates a change in metabolic pathways to protect breast cancer metastatic cells during transit from the primary tumor to the metastatic site.

BCL-2 immunostaining has been correlated with low AI, low histological grade, SR positivity, p53 expression, absence of c-ERBB-2, and better overall survival. It is surprising to find an inhibitor of apoptosis associated with better prognosis. Several explanations for these seemingly paradoxical results can be proposed: (1) regulation of BCL-2 expression by estrogen; (2) inhibitory effect of BCL-2 on cell cycle progression; (3) downregulation of BCL-2 by mutant p53; and (4) the presence of BCL-2 antagonists such as BAX or BAK, which negatively regulate its cytoprotective function. First, expression of BCL-2 is regulated by estrogens in mammary epithelial cells and ER-positive breast cancer cell lines. It gradually decreases during the development of breast cancers in relation with the loss of ER. Second, BCL-2 not only blocks cell death, but also has an independent inhibitory effect on cell division. The loss of BCL-2 can therefore enable high proliferation rates and high histological grade. Third, the inverse relationship between BCL-2 and p53 expression suggests that mutations in p53 could be related to the regulation of BCL-2 gene expression in breast cancers. Consistent with this hypothesis, transfection of mutant p53 into a wild-type p53 breast cancer cell line suppressed the expression of BCL-2. Finally, reduction in antiapoptotic BCL-2 can be compensated by a reduction in expression levels of the proapoptotic members BAX and BAK in some breast tumors. BAX immunostaining is associated with c-ERBB2 immunopositivity in invasive ductal carcinoma while reduced expression of both BCL-2 and BAX strongly correlates with the development of distant metastases. Reduction in BAK protein is associated with the conversion to hormone-independent ER-negative breast cancer and may play an important role in malignant progression by counteracting the reduced levels of BCL-2.

Anticancer therapies trigger apoptosis in part by modulating the expression of several members of the BCL-2 family and tipping the balance toward cell death. Paclitaxel acts by upregulating several proapoptotic BCL-2 proteins and downregulating antiapoptotic members. Similarly, doxorubicin causes a decrease in BCL-2 and an increase in BAX expression. Increased levels of BAX were correlated with a good response to chemotherapy in some studies. In the MCF-7 cell line, susceptibility

to drug-induced apoptosis was correlated with differential modulation of BAD, BCL-2, and BCL-XL protein levels, with Bad upregulation being an early indicator of a cell death outcome.

In summary, several members of the BCL-2 family regulate apoptosis in breast carcinomas. Alterations in their expression levels occur during tumorigenesis, tumor progression, and anticancer therapies. A reduction in BCL-2, BAX, and BAK, together with an upregulation of BCL-XL, are often seen in high-grade breast tumors. BCL-2 expression is associated with a number of favorable prognostic factors, whereas BAX expression seems to have a predictive value for positive response to chemotherapy in lymph node-negative breast cancer patients.

4. NONAPOPTOTIC TYPES OF CELL DEATH IN NORMAL AND NEOPLASTIC BREAST

Increasing evidence suggests that nonapoptotic modes of cell death also play a role in breast physiology and pathology. These distinct modes of cell death can occur concomitantly with apoptosis or provide alternative death pathways when apoptosis is impaired. Three types of nonapoptotic cell death have been described thus far in the normal and neoplastic breast: autophagy, necrosis, and entosis.

Autophagy (type 2 cell death) is a catabolic process of cellular “self-eating” involving the engulfment of the cell’s components (cytoplasm, organelles, and long-lived proteins) in double-membrane vacuoles (autophagosomes) and their degradation in lysosomes. Autophagy plays a dual role, pro-survival or pro-death, in both normal and neoplastic cells. It functions as a survival mechanism conferring temporary cytoprotection during nutrient starvation or metabolic stress. It helps maintain cellular homeostasis by preventing the accumulation of deleterious products and organelles and by supplying energy and amino acids through catabolism. Autophagy has also been described as a nonapoptotic type of cellular demise because extended autophagy ultimately results in type 2 programmed cell death. The role of autophagy, cell survival versus cell death, is both stimulus- and context-dependent. When needed, autophagic cell death can be used as an alternative to apoptosis to eliminate unwanted, damaged, or transformed cells. Regulators of apoptosis (e.g., BCL-2 family members, p53, AKT) also modulate autophagy, suggesting an intimate cross-talk between these two death pathways.

The role of autophagy in mammary development was shown by autophagosome detection during lumen

formation and early stages of involution. According to Fung and coworkers, autophagy during lumen formation would promote epithelial cell survival during anoikis. Autophagy observed in the involuting mammary tissue could be the natural cell defense against the transient nutrient and hormone deprivation after lactation and the energy supply for the apoptotic process.

Autophagy exerts a tumor suppressor function by preventing the accumulation of DNA-damaged cells or deleterious organelles such as reactive oxygen species-generating mitochondria. Defects in autophagy thus favor breast tumorigenesis by promoting genome damage and instability. Indeed, heterozygous disruption of the autophagy regulator *beclin-1* in mice results in reduced autophagy in vivo and development of various tumors. Monoallelic deletion in *BECLIN-1* is frequent in human breast carcinomas, which then express decreased beclin-1 protein levels when compared with adjacent normal tissue. Autophagy, which initially prevents tumorigenesis, plays an opposite survival role in later stages of breast cancer. It helps prevent the anoikis of metastatic breast tumor cells that lack ECM contact. Moreover, autophagy allows hypoxic inner regions within the tumor to survive metabolic stress. Defects in both autophagy and apoptosis in those hypoxic areas lead to cell death by necrosis.

Necrosis (type 3 cell death) is an irreversible inflammatory form of cell death induced by accidental cellular damage and characterized by the rupture of the plasma membrane and the release of the intracellular components to the surrounding tissues. Necrosis can also act as the ultimate backup mechanism for cell death when both apoptosis and autophagy are impaired. By provoking an inflammatory response similar to wound healing, necrosis stimulates angiogenesis and tumor growth. Necrosis is therefore a common feature of aggressive breast tumors associated with poor prognosis.

Apoptosis is not the only mode of cell death induced by anticancer therapies. Heterogeneous modes of cellular demise are observed when breast cancer cells are exposed to anticancer agents. Apoptosis prevails in most cases, but autophagy dominates in MCF-7 breast cancer cells treated with the antiestrogen tamoxifen, aromatase inhibitors, or new sesquiterpene analogs of paclitaxel. The mitotic inhibitor paclitaxel induces a biphasic death response in breast cancer cell lines: apoptosis at low concentrations and necrosis at high concentrations. The topoisomerase inhibitor camptothecin triggers both apoptosis and autophagy in MCF-7 cells. Silencing of *BID* in those cells led to a shift of cell death from apoptosis to autophagy, suggesting that BID could serve as

a molecular switch between these two types of cell death.

It has long been known that epithelial cells detached from their extracellular matrix undergo a process of apoptotic cell death called anoikis. Anoikis plays an important role in mammary gland development. This death mechanism intervenes in lumen clearing and involution and also prevents the formation of tumors that would fill the lumen. Inhibition of anoikis only delays lumen clearance in a 3D model of mammary acini, suggesting the existence of an alternate clearance mechanism in the detached cell population. This hypothesis was confirmed recently when Brugge and coworkers discovered another form of detachment-induced death named entosis. Entosis is described as a nonapoptotic cell death of matrix-detached cells involving cell-in-cell invasion followed by lysosomal degradation. Unlike dying cells that are cleared by phagocytosis, cells internalized by entosis are alive for a short term and can occasionally be released from their hosting cell. Entosis has been observed in normal breast epithelial cells in culture and metastatic tumor cells from fluid exudates. Further studies are required to better define the role of entosis in physiologic and pathological conditions such as breast cancer.

5. CONCLUSION

The breast is a hormone-responsive organ that completes most of its development after birth and to do so is highly dependent on effective apoptosis. In the normal breast, epithelial apoptosis promotes lumen clearance during ductal morphogenesis and removes excess secretory epithelium during involution. Apoptosis is also a prominent mechanism for tumor suppression and tumor regression after anticancer therapies. Deregulation of this death process is thus a hallmark of cancers such as breast carcinomas and has been the focus of intense studies.

In the normal and neoplastic breast, apoptosis regulation is multifactorial and dependent on both cell intrinsic and extrinsic factors. It is now widely accepted that the microenvironment and epithelium–stroma interactions in particular are key regulators of cell fate in vivo. These epithelium–stroma interactions are mediated by soluble secreted factors and cell-matrix adhesion. The ratio between prosurvival versus pro-death factors combined with cell adhesion status determine the cell fate of the mammary epithelium. Abnormal expression of cell adhesion molecules, stromal soluble factors, or their cell surface receptors are often observed

in breast carcinomas. These alterations ultimately lead to the deregulated hyperactivation of survival pathways, tumor growth, and evasion from apoptosis.

The survival and death signals transduced from the epithelial cell surface converge into the PI3K/AKT pathway. Activation of this pathway in breast carcinomas inhibits apoptosis and thereby significantly contributes to tumor progression and resistance to therapies. Altered expression of BCL-2 family members also participates in the deregulation of apoptosis in the neoplastic breast.

Nonapoptotic modes of cell death also play a role in breast physiology and pathology, as well as response to therapy. These distinct modes of cell death can occur concomitantly with apoptosis or provide alternative death pathways when apoptosis is impaired. The cross-talk and molecular switches between these different types of cell death require further investigation.

Emerging evidence suggests that normal stem cells and progenitor cells are likely targets for malignant transformation and that these transformed cells could function as cancer stem cells governing tumor growth. Candidate mammary cancer stem cells were isolated and characterized for the first time in 2003 by Clarke and coworkers. These cancer stem cells have several properties defined in the literature: high tumorigenicity, multipotency, self-renewal capacity, and high resistance to apoptotic stimuli. The current hypothesis is that this rare population of cancer stem cells would be at the origin of the disease and the likely cause for chemoresistance and cancer relapse. Research on those cells is in its infancy. Recent publications suggest that loss of p53 allows the expansion of presumptive cancer stem cells in mouse mammary tumors and in human breast cell lines. Further investigation of cell death regulation in those stem cells and their niche would help to better understand how breast cancer stem cells survive to conventional drug insult and how they can be targeted by new breast cancer therapies.

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Therapeutic Targeting Apoptosis in Female Reproductive Biology

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1. INTRODUCTION

The ovaries are major endocrine organs in females that, in mammals, serve two principal functions: (1) to produce a female germ cell (oocyte) that is capable of fertilization and successful embryonic development, yielding a viable, healthy offspring; and (2) to secrete a number of hormones that drive development of primary and secondary sex characteristics in the female and, during adulthood, prepare the uterus for establishment and maintenance of pregnancy.^{1,2} These functions are carried out by structures termed follicles,³ which are often referred to as the functional units of the ovaries. Each follicle is composed of an oocyte that is surrounded by one or more layers of somatic granulosa cells and, at more advanced stages of follicle development, theca cells. The granulosa and theca cells are responsible for much of the ovarian hormone production and support the maturation and growth of the enclosed oocyte. There are several different types of follicles present in the ovaries, which, depending on the size of the oocyte as well as the number of granulosa and theca cell layers, are classified as primordial (resting oocyte surrounded by a single layer of quiescent granulosa cells), primary (the first stage of immature follicles activated to initiate growth, characterized by oocyte enlargement and granulosa cell mitotic activity), secondary or preantral (larger maturing follicles with several layers of mitotically active granulosa cells, as well as some theca cells), and antral (mature follicles that have a fluid-filled cavity called an antrum and the ability to be ovulated in response to the pituitary gonadotropin, luteinizing hormone).^{1,2,3} The growth and maturation of a primordial follicle to the antral stage capable of ovulation can take weeks to months, depending on species,^{2,3,4} during which time the majority of follicles actually fail to mature and are

eliminated by a degenerative process involving apoptosis that is referred to as atresia.^{2,5,6,7,8,9}

During embryonic development, oocytes are derived from primordial germ cells (PGCs), which, through extensive mitotic divisions, establish the initial germ cell pool.^{10,11,12,13} Generally speaking, approximately halfway through gestation, the mitotically active PGCs in the embryonic female gonads, termed oogonia, enter meiosis and arrest at prophase of meiosis I to form oocytes (oogenesis). At this time, the oocytes are in dictyate arrest and begin the process of follicle formation (folliculogenesis) by recruitment of surrounding somatic cells that will eventually become granulosa cells.¹⁰ Throughout development and most of postnatal life, large numbers of germ cells and follicles are lost via a process that has many hallmark features of apoptotic cell death.^{5,6,7,8,9,14,15,16} Indeed, of the peak number of germ cells produced in the human ovaries (roughly 7×10^6 at week 20 of gestation¹²), greater than 99.9% will undergo cell death at some point before complete exhaustion of the oocyte-containing follicle pool at menopause.^{8,17} Thus the “normal” fate of an oocyte is death, not survival. Because of this ongoing and extensive loss, the ovaries have provided an excellent model system for understanding the process of physiologic cell death. In fact, one of the first detailed documentations of apoptosis (long before the term was coined) was made with rabbit ovaries by Flemming in 1885,¹⁸ who detailed the microscopic features of dying granulosa cells in antral follicles undergoing atresia. Flemming termed this process *chromatolysis* on the basis of his observations of the condensation and ultimate disintegration of nuclear chromatin in the dying cells. In fact, Flemming's descriptions of chromatolytic cell death in 1885 bear close parallels to the morphological features of apoptosis detailed by Kerr, Wyllie, and Currie when the term was first coined

in 1972.¹⁹ As limited methods were available in the late 1800s and early 1900s to study oocyte and granulosa cell death in detail, the apoptotic nature of these processes was not revisited until about 100 years later. Today, a wealth of morphological, biochemical, and genetic evidence is available demonstrating that controlled physiologic cell death occurs at relatively high levels in both fetal and postnatal ovaries.^{7,8,9,14,15,16,20,21,22,23,56}

As a consequence of the continuous depletion of female germ cells, natural cessation of female reproductive function occurs around mid-life (termed the menopause in humans),^{14,23} coincident with essentially complete depletion of the oocyte-containing follicle pool.^{13,14,15,16} This key event in the life of females has many ramifications. First, functional ovarian lifespan determines the maximum age at which women are able to conceive naturally and via assisted reproductive technologies.^{24,25,26} Because increasing numbers of women are electing to postpone their childbearing to ages of suboptimal fertility (i.e., late 30s and 40s), the decline in ovarian function with age has become an increasingly challenging issue for many women in Western societies.^{27,28} In addition to fertility issues, ovarian failure with age also results in other physiologic complications.^{14,23} For example, as a consequence of complete follicle depletion, the lack of cyclic ovarian hormone production results in widespread endocrine changes that are well-known risk factors for development of several age-related health issues in women, such as osteoporosis, heart disease, and cognitive decline. In turn, the development of methods to delay age-related oocyte depletion²⁹ could have enormous implications for improving the quality of life in aging women.

It is important to emphasize that in addition to the normal physiologic loss of female germ cells, many women experience an accelerated loss of oocytes resulting from exposure to pathological insults.^{30,31,32,33,34,35} For the purposes of discussion, these insults can be categorized as either those associated with clinical care for various diseases (e.g., chemotherapy and radiotherapy)^{30,31,32} or those arising from the environment (e.g., man-made chemicals and industrial by-products).^{33,34,35} Interestingly, a wealth of information from both animal and human studies has demonstrated that, contrary to what one might expect of the response to pathological insults, gene-regulated death of germ cells and follicles appears to play a primary role in the premature ovarian failure that is often observed.^{7,8,9,36,37,38,39,40,41} As will be addressed in more detail in the following sections, this information has provided a solid basis to explore ways to circumvent oocyte loss resulting from these types of

insults as a novel means to sustain ovarian function and fertility in women treated for cancer.

2. DETECTING CELL DEATH IN THE FEMALE GONADS

Initially, the occurrence of physiologic cell death in mammalian ovaries was derived from morphological characterization of dying oocytes and granulosa cells, followed by biochemical identification of fragmented chromosomal DNA.^{5,6,14,15,18,20,21,22} At present, morphological evaluation of ovarian tissue sections remains a standard, if not preferred, method for the detection of cell death in oocytes and granulosa cells. Typical morphological features of dying germ cells in embryonic and postnatal ovaries include chromatin condensation, cytoplasmic shrinkage and vacuolization, crowding of organelles, surface protuberances, cytoplasmic and nuclear condensation and fragmentation, and nucleolar segregation. It bears mentioning that the application of various biochemical and molecular biological techniques to detect apoptosis, including the terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) assay, immunostaining with active caspase-specific antibodies, and phosphatidyl serine exposure on the outer leaflet of plasma membrane, have produced conflicting results with respect to their detection in dying female germ cells.^{42,43,44} This likely stems from differences in the models employed by different investigators to trigger oocyte death, the maturational stage of the oocytes being studied, and the developmental window (i.e., fetal vs. postnatal life) under investigation. Nevertheless, there is agreement, and genetic evidence, that many genes and signaling pathways classically associated with apoptosis, such as the sphingomyelin pathway, death receptors (most notably Fas/CD95), Bcl-2 family members, and caspases,^{7,8,9,16,23,36,37,38,39,40,41,45,46,47,48,49,50} play instrumental roles in the regulation of germ cell and follicle depletion under most situations. Given this and the morphological information available, for this chapter we use the term apoptosis when discussing instances of female germ cell death known to be regulated by these gene-driven pathways, rather than attempting to use multiple terms (e.g., programmed cell death, autophagy, necroptosis) to refer to these various examples of controlled cell death.

3. OCCURRENCE AND REGULATION OF CELL DEATH IN THE OVARIES

Cell death has been detected in nearly all cell types within the mammalian ovaries, although the vast

majority of studies have focused on either germ cells (PGCs, oogonia, or oocytes) or granulosa cells.^{7,8,9} With respect to the germline, apoptosis occurs in this cell lineage very early in embryonic development.^{7,8,9,14,15} For example, PGCs can first be identified as alkaline phosphatase-positive cells posterior to the primitive streak region of the gastrula.^{51,52} From this region, PGCs migrate through the hindgut and dorsal midline region and then laterally into the genital ridges of the embryo.^{52,53,54} Extensive evidence, primarily from rodent studies, indicates that PGCs that fail to reach the developing gonads during this migration are eliminated through Bax-dependent apoptosis in extragonadal sites because of a lack of tropic factors (primarily *Steel* factor or stem cell factor) that support their survival.^{55,56,57,58,59} Those PGCs that successfully reach and colonize the genital ridges undergo successive mitotic divisions as oogonia that give rise to peak numbers of germ cells, some of which enter meiosis and arrest in the first meiotic prophase (oocytes).^{1,2,10,11,12,13} However, vast numbers of oogonia and oocytes also die during this time, such that only about one-third of the peak number of germ cells produced by the female survives shortly after birth as follicle-enclosed oocytes.^{7,8,9} Experimental evidence indicates that at least two principal pathways trigger this developmental germ cell death in the female.⁸ One pathway is activated as a response to an inadequate supply of external cytokine survival signals and involves the sphingomyelin pathway, the Bcl-2 family of proteins and caspases (primarily caspase-9 and caspase-2).^{37,39,57,58,60,61,62} The second pathway appears to be cytokine- and Bax-independent and is triggered by defects in meiotic initiation or arrest.⁶² It should be mentioned that in *Drosophila*, incomplete cytokinesis during germ cell mitosis leads to the formation of germ cell cysts – structures in which several germ cells remain connected to each other by intercellular bridges.⁶³ During germ cell differentiation, every cell in the cyst but one undergoes apoptosis, with the dying germ cells serving to provide the sole remaining germ cell with nutrients, macromolecules, and organelles necessary for further development. Although recent studies have identified the formation of germ cell cysts in rodent ovaries during development, only a few germ cells in the cyst appear to activate apoptosis.⁶⁴

Oocytes continue to die throughout postnatal life as well. Of the approximately 1 to 2×10^6 oocytes present in humans at birth,¹² the pool of oocyte-containing follicles declines to less than 3×10^5 by puberty,¹³ and fewer than 1×10^3 oocytes remain just before menopause.¹⁷ Because only one egg on average reaches the appropriate maturity to be ovulated each month, at most an

adult woman can ovulate 400 oocytes in total during her entire reproductive years. The vast majority ($>99.9\%$) of oocytes are lost through follicle atresia. Atresia can apparently occur at any point during the maturation of follicles from the resting (primordial) to mature (antral) stage, although histological evidence indicates that most follicles die during transition from the primary to preantral, and from the preantral to antral, stages of development.⁶⁵ Interestingly, histological data from studies of human and rodent ovaries indicate that immature follicle atresia (primary, early preantral) is probably triggered by initial death of the oocyte, whereas atresia during later stages of follicle maturation (late preantral, antral) is a consequence of granulosa cell apoptosis being activated before discernible changes in the oocyte.^{7,8,9,65} Regardless, the end result is the same – irreversible loss of that oocyte and follicle from the available pool.

The signals and genes that serve as determinants of germ cell and granulosa cell fate in the ovaries have been a subject of considerable research interest since the early 1990s. Through a combination of in vitro cell and organ culture models, coupled with extensive validation in vivo (primarily in rats and mice), a complex network of converging receptor-mediated survival and death signals, coupled to activation of a variety of intracellular signaling cascades ranging from ceramide generation to phosphatidylinositol-3-kinase/Akt, have been described in detail.^{8,9,39,60} Further, the availability of mutant mouse lines lacking critical regulators of apoptosis – most notably, Bcl-2 family members and caspases – has had a tremendous impact on the field of ovarian cell death research, as correlative gene expression studies could be tested for their functional relevance to various models of oocyte and follicle loss.^{7,8,9,23} As a result, a number of pathways and genes have been identified as absolutely critical for apoptosis to occur in oocytes and granulosa cells, and cell lineage-specificity for the necessity of certain proteins, such as caspase-3, has been also been demonstrated.^{7,8,9,23,46} However, because this information has been recently reviewed in detail elsewhere,^{7,8,9,23} we devote the remainder of this chapter to a discussion of two specific examples in which the ability to manipulate apoptosis has considerable clinical potential for improving women's health.

4. APOPTOSIS AND FEMALE REPRODUCTIVE AGING

As discussed previously, in human females, less than 5% of the peak germ cell population produced during development survives at puberty, and this number continues to decline during adulthood to the point of exhaustion at approximately age 50 years, driving the menopause.¹⁷

Hence the ovaries represent one of the first major organ systems to fail with advancing chronological age, and their failure marks a transitional period characterized by increased risk for development of a spectrum of debilitating health complications in the ensuing years.²⁴ Similar reductions in oocyte numbers have been reported in female mice throughout postnatal life, with complete depletion of the oocyte pool also noted several months before death due to chronological age.^{7,8,9,66} The postnatal loss of oocytes and follicles through atresia is mediated in large part via apoptosis, a point most clearly established by studies of mutant female mice lacking expression of the proapoptotic Bax protein.⁶⁷ In these investigations, it was shown that oocytes of Bax-deficient female mice are resistant to developmental cues responsible for triggering apoptosis, thus leading to a reduced incidence of immature follicle atresia and a dramatic extension of functional ovarian lifespan into advanced chronological age.²⁹ In addition to providing the first vertebrate animal model that fails to undergo its equivalent of menopause with age, Bax-deficient mice validated a critical concept in mammalian female reproductive biology – experimentally increasing the size of the immature follicle pool can extend the functional lifespan of the ovaries well past their normal time of senescence. Of further note, oocytes in the ovaries of very old Bax-deficient females remain fully capable of generating viable offspring if the aged ovarian tissue is transplanted into young adult recipient females.²⁹

Subsequent characterization of aging Bax-deficient females revealed that sustaining ovarian function through maintenance of the follicle pool yielded a number of significant health benefits.⁶⁸ For example, the age-related onset of bone and muscle loss, excess fat deposition, alopecia, cataracts, deafness, increased anxiety, and selective attention deficit observed in aging wild-type females was attenuated or absent in Bax-deficient female siblings. Further, maintaining ovarian function with age did not increase the incidence of cancer in any tissues, particularly those responsive to ovarian steroid hormones. Somewhat surprisingly, however, the reduced incidence or severity of age-related health complications noted in aged Bax-deficient females did not equate to increased longevity.⁶⁸ Nevertheless, these findings support the idea that significant improvements in the overall quality of life in aging females can be achieved by approaches that sustain follicle numbers and, consequently, ovarian function. With that said, it is important to keep in mind that extrapolation to humans is difficult to foresee given that one cannot inactivate specific genes (e.g., *Bax*) in humans to achieve a similar outcome. Accordingly,

some consideration must be given to exploring and developing more practical methods of extending the fertile lifespan of females that could be reasonably viewed as amenable for translation and human application at some point in the future. Some progress has been made in this regard over the past few years.

The first example of this revolves around a series of recent findings challenging the dogma that, unlike males, mammalian females do not share the luxury of a renewable germ cell pool during postnatal life. For more than five decades, a central underpinning of mammalian reproductive biology has been that the reserve of oocytes set forth at birth cannot be replenished or replaced.⁶⁹ However, experimental results from an increasing number of labs have challenged this belief, thus opening the door to a number of new possibilities to consider with respect to exploring the role that adult stem cells may play in oogenesis, fertility, and ovarian aging in females.^{70,71,72,73,74,75,76,77,78} In turn, efforts are now needed to determine whether and when apoptosis of these stem/progenitor cells, or the cells comprising the microenvironments that support them, occurs in the lifespan of the female and how this process plays into ovarian failure with age. Some insights into this may come from transplantation models, as exemplified by very recent studies with mice showing that infusion of bone marrow-derived stem cells from young donor females into aging recipients remarkably extends their reproductive lifespan.⁷⁶ Although the mechanisms underlying this outcome remain to be identified, it is possible that the “young” stem cells have replaced missing stem cells lost through apoptosis as a result of replicative arrest associated with advancing age.

The second example is related to the impact of dietary caloric intake on fertility in females with age. Although there are several historical reports documenting a positive effect of caloric restriction (CR) on reproductive performance in rodents, much of the past work evaluating the effect of CR on long-term fertility in female mice initiated CR at or before weaning, which has obvious limitations in the context of human application.^{79,80,81} However, recent studies have shown that female mice placed on a moderate CR protocol during adulthood have increased numbers of oocytes later in life compared with age-matched ad libitum (AL)-fed controls, and this in turn is associated with a dramatic extension of reproductive lifespan.⁸² Although the impact of CR on the incidence of germ cell apoptosis was not assessed in this study, it is logical to assume that the expanded oocyte reserve seen in CR females during adulthood reflects a reduced rate of postnatal loss via atresia. Further, although the feasibility of applying adult-onset CR

protocols in humans for the sole purpose of extending ovarian lifespan is debatable, current efforts to develop small-molecule CR mimetics^{83,84,85,86,87} may provide an alternative strategy to achieve this outcome in aging females.

5. ANTIAPOPTOTIC AGENTS AND FERTILITY PRESERVATION FOR CANCER SURVIVORS

Recent estimates from the American Cancer Society indicate that ~2% of all young girls and reproductive-age women in the United States are diagnosed with cancer annually.⁸⁸ The majority of these patients will undergo some type of treatment protocol involving the administration of cytotoxic drugs and/or radiation therapy in an attempt to eradicate their cancer. Unfortunately, because it is currently not possible to target only the cancerous cells, side-effect damage to healthy tissues is an inevitable consequence of these treatments. In this regard, ovarian follicles are remarkably vulnerable to ionizing radiation and many chemotherapeutic drugs.^{8,9,30,31,32,89,90,91,92} Indeed, accelerated loss of oocytes, premature ovarian failure, and infertility are well-recognized, and undesired, outcomes in young girls and reproductive-age women treated for cancer. Yet, despite the significance of these complications to the quality of life of cancer survivors and the fact that cancer treatment-induced loss of ovarian function has been recognized since the 1950s, relatively little has been done to protect the ovaries from these insults. Hence female cancer patients are forced to rely on assisted reproductive technologies before and after their treatment for a chance at fertility.^{93,94} However, oocyte retrieval for either cryopreservation or in vitro fertilization requires extensive hormonal manipulation, which may not be ideal for patients combating cancers that are aggravated by exposure to estrogens (i.e., breast cancer) or requiring a rapid initiation of treatment.^{93,94} In addition, many assisted reproductive technologies are not suitable for prepubertal girls or single (unmarried) women and, perhaps more importantly, none prevent the premature loss of ovarian function and the ensuing health problems that arise from it.

However, progress toward preserving ovarian function and fertility in female cancer patients without the use of assisted reproductive technologies has been made in the recent years. This stems primarily from a pivotal study published in 1997, which showed that the mechanism of anticancer therapy-induced oocyte loss involves apoptotic cell death as opposed to uncontrolled necrosis.³⁶ Thus the possibility of preventing follicle death caused by chemo- or radiotherapy via a targeted

inhibition of apoptosis emerged as a testable hypothesis. Importantly, this work was built on a platform of substantial information already regarding the specific genes and pathways involved in the activation and execution of cell death in oocytes and ovarian follicles under normal physiologic conditions.^{7,8,9} Drawing parallels from this body of work, extensive thought was given to the many potential targets for therapeutic development. The most logical to test were the earliest steps leading to apoptosis commitment, as inhibiting events after mitochondrial destabilization delays apoptosis but the cells nonetheless eventually die via a process more akin to necrosis.

A key mediator of mitochondrial permeabilization in many types of cells is the proapoptotic Bax protein.⁹⁵ Experiments with gene mutant mice also identified Bax as being absolutely required for oocyte loss under normal physiologic conditions, as well as under a host of pathological situations associated with premature ovarian failure.^{29,36,40,41} However, the lack of a small-molecule Bax inhibitor at that time necessitated consideration of other, perhaps earlier, steps in the signaling cascade leading to apoptosis commitment. In this regard, generation of ceramide via acid sphingomyelinase (ASMase)-mediated membrane hydrolysis was subsequently reported as an initiator of proapoptotic signaling in oocytes during development.^{36,39} Ceramide is sphingolipid produced in many cell types after exposure to stress.⁹⁶ Ceramide can either signal for apoptosis, or it can, if the damage to the cell is not irreparable, be metabolized to sphingosine, which serves as a precursor for the generation of sphingosine-1-phosphate (S1P). In some cells, S1P can effectively counteract the proapoptotic effects of ceramide, leading to enhanced cell survival.⁹⁷ In initial studies conducted to test whether S1P could protect oocytes from death caused by cancer treatments, it was shown that the majority of oocytes cultured in vitro in presence of S1P were insensitive to the proapoptotic effects of the chemotherapeutic drug, doxorubicin.³⁶ Additional in vitro studies with oocytes harvested from female mice lacking expression of ASMase supported that the generation of ceramide from sphingomyelin was an early critical step in chemotherapy-induced oocyte death.³⁹ In vivo studies with mice soon followed, confirming that delivery of S1P to the ovaries of adult female mice 2 hours before irradiation maintained their ovarian follicle reserves.³⁹ Importantly, these radioprotected females were able to give birth to healthy and cytogenetically normal offspring,⁹⁸ indicating that the targeted suppression of female germline cell death after exposure to a cytotoxic insult does not simply result in the accumulation of damaged or “undead” oocytes in the ovaries.

Over the past few years, the wealth of information obtained from nearly 10 years of work with rodent models demonstrating the efficacy of S1P in preserving ovarian function and fertility of adult females exposed to conventional cancer treatments^{36,39,98,99,100,101} has served as a basis for testing the translational feasibility of using S1P as a fertility preservation agent in primates. This work is important for two principal reasons. The first and foremost is related to the very real possibility that the outcomes obtained with drug studies using rodent models will not be observed in primates as a result of species differences in drug bioactivity, metabolism, and the like. The second hurdle is one of an anatomical nature because primate ovaries are not enclosed within bursal sacs, as is the case with rodent ovaries. This is a key aspect in determining the potential utility of any antiapoptotic compound for the purpose of protecting the ovaries, because systemic availability of the compound could also protect the tumor targeted for destruction. In mice and rats this can be, and in fact was, circumvented by direct intrabursal injection of S1P before irradiation.^{36,98} Without a bursa, primate ovaries are not amenable to this type of localized drug delivery. However, using an intraovarian catheter-osmotic minipump system developed specifically for this purpose, very recent studies have reported that direct and controlled long-term delivery of S1P or the long-acting S1P mimetic FTY720 to the ovaries of adult female primates can be successfully achieved. Moreover, this approach was reported to protect monkey ovaries from the damaging effects of radiotherapy, leading to a maintenance of natural fertility and birth of offspring free of anatomical or cytogenetic defects.¹⁰² These encouraging findings provide important translational proof-of-concept that targeted antiapoptotic therapies can be used to preserve ovarian function and fertility in primates exposed to devastating cancer treatments.

6. CONCLUDING REMARKS

For many years, the field of apoptosis flourished by virtue of the fact that new regulatory genes and pathways were discovered on almost a daily basis. Many additional years have been spent attempting to integrate all of this information into a working generic blueprint for how apoptosis is activated and executed in various cell types and how these events are influenced by the surrounding environmental cues being constantly interpreted by the cell.^{103,104,105,106,107} With the assembly of this blueprint nearing completion, the new challenge faced by those in this field revolves around addressing

the question of how this information could actually be used for combating diseases and improving organ or tissue function in humans.^{108,109,110} Our goals here were to concisely summarize a few of the highlights of cell death research in the field of female reproduction, and in particular ovarian biology, over the past 20 years and to provide examples of how scientists in this field are attempting to meet the challenge of translating basic science findings to clinical medicine. Although some of these examples are clearly early stage, the progression of work to validate S1P as an ovarian protectant and fertility preservation agent for female cancer patients undergoing cytotoxic treatments clearly shows that such translational work with antiapoptotic compounds is feasible. Although the true measure of success of this approach awaits the final outcome of a future clinical trial in humans, as little as 10 years ago this line of thinking was viewed by many as not likely to succeed. However, the importance of the goal – improving the quality of life for female cancer survivors – far outweighed any resistance met, eventually leading the field of reproductive medicine to a point never before reached: protection of primate ovaries from 15 Gy of radiation using a small antiapoptotic molecule.¹⁰² So, does a delay of age-related ovarian failure and menopause, which has considerable ramifications for improving the quality of life in aging women, really seem that unattainable? If history is our best teacher, then the answer is no, especially considering the striking observations already made with aging Bax-deficient female mice.^{29,68} The challenge will be how to take what has been learned from these types of rodent studies and devise clinically amenable strategies for sustaining the oocyte and follicle pool in women as they age.

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Apoptotic Signaling in Male Germ Cells

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ABSTRACT

Programmed germ cell death (apoptosis) is conspicuous during normal spermatogenesis and serves as a quality control system for the production of normal sperm. Deregulation of germ cell death is associated with defective spermatogenesis and impaired fertility. Mitochondria-dependent intrinsic pathway signaling constitutes a critical component of apoptotic signaling in male germ cells across species. However, the regulation of germ cell apoptosis may vary depending on the nature of the apoptotic stimulus and can be triggered by more than one pathway. Activation of p38 mitogen-activated protein kinase (MAPK) and induction of inducible nitric oxide synthase are critical for activation of the intrinsic pathway signaling in male germ cells. In addition, there is increasing evidence that caspase-2 is an upstream activator of the p38 MAPK and nitric oxide-mediated intrinsic pathway signaling. This chapter focuses on the recent progress in our understanding of the regulation of germ cell apoptosis in the testis.

1. INTRODUCTION

Spermatogenesis is a dynamic process in which stem spermatogonia, through a series of events, become mature spermatozoa and occurs continuously during the reproductive lifetime of the individual (Russell et al., 1990). Stem spermatogonia undergo mitosis to produce two types of cells: additional stem cells and differentiating spermatogonia; the latter undergo rapid and successive mitotic divisions to form primary spermatocytes. The spermatocytes then enter a lengthy meiotic phase as preleptotene spermatocytes and proceed through two cell divisions (meiosis I and II) to give rise to haploid spermatids. These in turn undergo a complex process of morphological and functional differentiation resulting in the production of mature spermatozoa. All these phases are supported by and are dependent on an intimate interaction between germ cells and the Sertoli cells (Russell et al., 1990). Not all germ cells, however, achieve

maturity, and such spontaneous death of certain classes of germ cells by apoptosis appears to be a constant feature of normal spermatogenesis. Increased germ cell death by apoptosis can be triggered by various regulatory stimuli, including testicular hyperthermia or experimental male hormonal contraceptive (Sinha Hikim and Swerdloff, 1999; Sinha Hikim et al., 1999; Sinha Hikim et al., 2003a). Disruption of this orderly process of germ cell death is associated with several impairments of spermatogenesis and fertility (Dunkel et al., 1997; Yamamoto et al., 2001; Pentikaainen, Dunkel, and Erkkila, 2003; Shaha, 2007; Wang et al., 2007). This chapter focuses on unique aspects of genetic regulation of spermatogenesis and highlights the signal transduction pathways inducing male germ cell apoptosis across species.

2. TESTICULAR GERM CELL APOPTOSIS HAS MANY UNIQUE REGULATORY GENES

The most important insight into the intracellular mechanisms that control male germ cell apoptosis comes from studies using genetically altered mice either overexpressing or harboring a null mutation of specific genes. Male germ cell apoptosis, like that of other cell systems, is a genetically driven process (reviewed in references Sinha Hikim and Swerdloff, 1999; Matzuk and Lamb, 2002; Baum, St. George, and McCall, 2005; Sinha Hikim, Swerdloff, and Wang, 2005). Null mutation of some genes, which are expressed in many tissues, including the testis, can accelerate germ cell apoptosis and cause specific defects in spermatogenesis. Many genetic alteration impair spermatogenesis without affecting other systems including oogenesis.

A number of examples exist whereby gene deletion results in specific defects in spermatogenesis. Elimination of Bcl-w leads to male sterility with no discernible

effects on most of the systems, including the female reproductive function (Ross et al., 1998; Print et al., 1998). Mutant animals have a block in the later phases of spermatogenesis and exhibit progressive depletion of germ cells through accelerated apoptosis to a Sertoli-cell-only phenotype by approximately 6 months of age followed by loss of Sertoli cells. Given that BCLW is expressed in the elongated spermatids and in Sertoli cells (Ross et al., 1998), it is likely that death of late spermatids is due to the absence of BCLW function in those germ cells, whereas depletion of the entire germline in adults reflects the loss of BCLW function in the Sertoli cells. Targeted gene disruption of Hsp 70-2 results in failed meiosis and increased germ cell apoptosis in males (Dix et al., 1996); however, neither meiosis nor fertility is affected in female Hsp 70-2^{-/-} mice. Likewise, targeted disruption of the mouse homolog of *Drosophila* Vasa (Mvh) results in male sterility as a result of massive increase in germ cell apoptosis involving meiotic and postmeiotic germ cells, with no adverse effects on female fertility (Tanaka et al., 2000). Inactivation of HR23B ubiquitin-conjugating DNA repair enzyme causes male sterility due to increased germ cell loss with no effect on female fertility (Roest et al., 1996). In contrast to a lethal phenotype of TATA-binding protein-related factor 2 (TRF2) inactivation in *Caenorhabditis elegans* and *Xenopus laevis*, TRAF2-deficient mice are viable and show no apparent abnormalities in major organs (Zhang et al., 2001). However, TRAF2^{-/-} male mice are sterile because of a severe defect in spermatogenesis, whereas female TRAF2-deficient mice are fertile and produce normal average litter size (Zhang et al., 2001). Female transgenic mice with selective over-expression of BCL-2 in the somatic cells of the ovary exhibit decreased follicle apoptosis, enhanced folliculogenesis, larger litter size, and increased susceptibility to germ cell tumorigenesis (Hsu et al., 1996). In striking contrast, selective over-expression of BCL-2 in the somatic cells of the testis exhibits variable impairment of spermatogenesis (Yamamoto et al., 2001). Apaf-1 deficient mice closely resemble caspase-3 and caspase-9 knockout mice and die perinatally with severe craniofacial abnormalities, brain overgrowth, and reduced apoptosis in the central nervous system (Cecconi et al., 1998; Yoshida et al., 1998; Honarpour et al., 2000). Of further interest, approximately 5% of the Apaf-1 knockouts survive to adulthood, and, in contrast to the nonsurviving mutants, the survivors lack brain pathology but exhibit profound defects in spermatogenesis (Honarpour et al., 2000). The ablation of the Bax gene by homologous recombination also results in male sterility due to accumulation of atypical premeiotic germ cells but with accelerated

apoptosis of mature germ cells, leading to complete cessation of sperm production (Knudson et al., 1995). Conversely, thymocytes and B cells display hyperplasia, and Bax^{-/-} ovaries accumulate an excess of granulosa cells and primary as well as primordial follicles as compared with those of wild-type mice (Knudson et al., 1995; Perez et al., 1999). Thus the loss of Bax results in hyperplasia or hypoplasia, depending on the cellular context. Taken together, these studies suggest that even the same molecule can play different roles in regulation of male germ cell apoptosis. More detailed analysis of the available mutant models with additional manipulation (using different apoptotic inducers) are needed to understand the precise signal transduction pathways regulating testicular germ cell apoptosis.

3. MODELS TO STUDY TESTICULAR GERM CELL APOPTOSIS

3.1. Murine models

The issue of experimental models is important from a clinical standpoint because many aspects of signal transduction pathways regulating human testicular germ cell apoptosis are not amenable to study directly. Accordingly, we took advantage of various well-characterized model systems. Programmed germ cell death can be triggered by a variety of apoptotic stimuli, such as mild testicular hyperthermia, and deprivation of gonadotropins and testicular testosterone (T) by gonadotropin-releasing hormone antagonist (GnRH-A) or by exogenous administration T, Sertoli cell toxicant, and chemotherapeutic agents (reviewed in Sinha Hikim et al., 2003a). We previously reported that selective deprivation of gonadotropins and testicular T is followed by a stage-specific apoptosis of germ cells involving preleptotene and pachytene spermatocytes and round and elongated spermatids at mid (VII and VIII) stages (Sinha Hikim et al., 1995; Sinha Hikim et al., 1997). In subsequent studies, we demonstrated that transient heat exposure also induces stage-specific activation of apoptosis, but at different stages of spermatogenic cycle (Lue et al., 1999; Lue et al., 2000; Sinha Hikim et al., 2003b). In striking contrast to hormone deprivation model, a transient exposure of the testes to heat (43°C for 15 minutes) induces germ cell apoptosis exclusively at early (I–IV) and late (XII–XIV) stages. Pachytene spermatocytes and early spermatids (steps 1–4) at stages I through IV and pachytene, diplotene, and dividing spermatocytes at stages XII through XIV are most susceptible to heat. As depicted in Figure 25-1, the vulnerability of germ cells to apoptosis in these two paradigms is different. Similar

Stage- and cell-specific activation of apoptosis

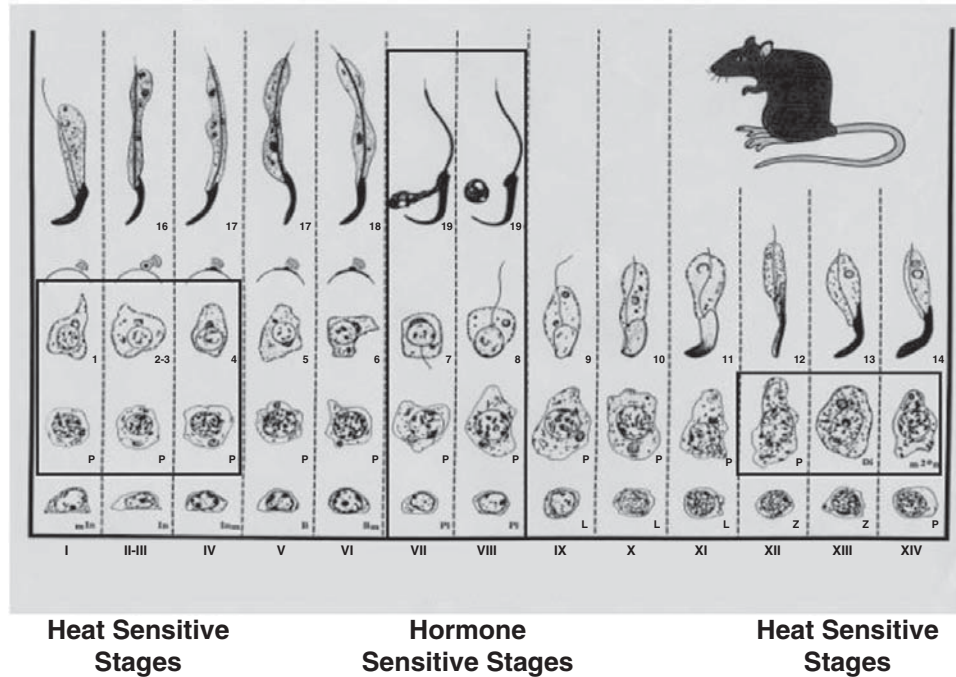


Figure 25-1. Stage-specific activation of male germ cell apoptosis. Diagrammatic representation of the seminiferous epithelial cycle in the rat to illustrate the stage-specific activation of germ cell apoptosis triggered by deprivation of the gonadotropic support or by mild testicular hyperthermia. The columns numbered with Roman numerals show the various cell types present at each stage (Russell et al., 1990). Different types of A-spermatogonia are not indicated in the cycle map. Early deprivation of gonadotropins and intratesticular T induces apoptosis at stages VII and VIII (designated as hormone-sensitive stages) involving preleptotene (PL) and pachytene (P) spermatocytes and round (steps 7 and 8) and step 19 spermatids. In striking contrast, testicular hyperthermia induces germ cell apoptosis at early and late stages (designated as heat-sensitive stages). P spermatocytes and early spermatids (steps 1–4) at stages I through IV and late spermatocytes at stages XII through XIV are most susceptible to heat.

stage-specific activation of germ cell apoptosis triggered by testicular hyperthermia or hormone deprivation has also been validated in mouse models (Sinha Hikim et al., 2003a; Sinha Hikim et al., 2003b; Sinha Hikim et al., 2005; Vera et al., 2006). However, we had to add flutamide to GnRH-A for effective induction of male germ cell apoptosis in mice after hormone deprivation (Sinha Hikim et al., 2005; Vera et al., 2006).

3.2. Primate models

We have extended our experimental paradigm from rodents to monkeys (Lue et al., 2006; Jia et al., 2007) and more recently in humans (Wang et al., 2007) and demonstrated that, indeed, germ cell apoptosis plays an important role in the organized regression of spermatogenesis after hormone deprivation and/or testicular hyperthermia. We have further initiated in vitro studies in humans to elucidate the molecular mechanism of germ cell apoptosis (Vera et al., 2006). Induction of germ cell

apoptosis can be readily achieved by culturing segments of seminiferous tubules under serum-free conditions.

We took advantage of these different but complementary models for induction of testicular germ cell apoptosis to elucidate the key signal transduction pathways for male germ cell apoptosis across species.

3.3. Pathways of caspase activation and apoptosis

As depicted in Figure 25-2, the signaling events leading to apoptosis can be divided into two major pathways, involving either mitochondria or death receptors (Tilly 2001; Danial and Korsmeyer, 2004; Youle and Strasser, 2008). The mitochondria or the intrinsic pathway for apoptosis involves the release of cytochrome c into the cytosol, where it binds to apoptotic protease activating factor-1 (Apaf-1), resulting in the activation of the initiator caspase-9 and the subsequent proteolytic activation of the executioner caspases-3, -6, and -7. Members of the BCL-2 family of proteins play a major role in governing

this mitochondria-dependent apoptotic pathway, with proteins such as BAX functioning as inducer and proteins such as BCL-2 as suppressor of cell death. Additionally, SMAC (second mitochondria-derived activator of caspases), also known as DIABLO, is released from mitochondria into the cytosol after apoptotic stimuli and promotes apoptosis by antagonizing inhibitor of apoptosis proteins (IAPs). The death receptor or the extrinsic pathway for apoptosis involves ligation of the death receptor (such as FAS) to its ligand, FASL. Binding of FASL to FAS induces trimerization of FAS receptors, which recruit Fas-associated death domain (FADD) through shared death domains (DD). FADD also contain a death effector domain (DED) in its N-terminal region. FAS/FADD complex then binds to the initiator caspase-8 or -10, through interactions between DED of the FADD and these caspase molecules. Cross-talk between these pathways does occur at some levels. In certain cells, caspase-8 through cleavage of BID, a proapoptotic BCL-2 family member, can induce cytochrome c release from mitochondria in FAS-mediated death signaling. Both these pathways converge on caspase-3 and other executioner caspases and nucleases that drive the terminal events of programmed cell death.

3.4. Apoptotic signaling in male germ cells

Mitochondria-dependent intrinsic pathway signaling is a key pathway for male germ cell apoptosis.

In earlier studies, using a rat model of testicular hyperthermia, we characterized the key molecular components of the effector pathways leading to caspase activation and increased germ cell death in the testis (Sinha Hikim et al., 2003b). Short-term exposure (43°C for 15 minutes) of the rat testis to mild heat results, within 6 hours, in stage- and cell-specific activation of germ cell apoptosis. Initiation of apoptosis was preceded by a redistribution of BAX from a cytoplasmic to a paranuclear localization in heat-susceptible germ cells and elevated levels of BCL-2 in the mitochondria. The relocation of BAX is accompanied by cytosolic translocation of cytochrome c and is associated with activation of the initiator caspase-9 and the executioner caspases-3, -6, and -7 and cleavage of poly (ADP-ribose) polymerase (PARP). Collectively, these data suggest the involvement of the mitochondria-dependent pathway for heat-induced male germ cell apoptosis.

To characterize the involvement of the intrinsic pathway signaling for induction of apoptosis in our hormone deprivation model, groups of adult male rats were given a daily injection of vehicle for 14 days or GnRH-A acyl-line at a dose of 1.6 mg/kg of body weight for 2, 5, and

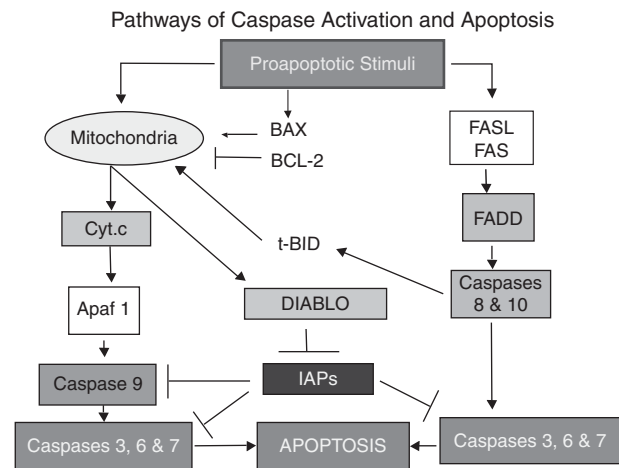


Figure 25-2. Intrinsic and extrinsic pathways for caspase activation and apoptosis. The mitochondria or the intrinsic pathway of apoptosis involves release of cytochrome c (Cyt c) from mitochondria into the cytosol, where it binds to Apaf 1, resulting in the activation of caspase-9 and the subsequent activation of the executioner caspases-3, -6, and -7. The BCL-2 family of proteins usually governs the intrinsic pathway for apoptosis. The extrinsic or the death receptor pathway involves ligation of FAS to FASL, resulting in the activation of a different set of initiator caspases, namely caspase-8 and -10, through interactions between death domains and death effector domains of an adapter molecule such as FADD and these caspases. Both these pathways eventually converge on caspase-3 and other executioner caspases that drive the terminal events of programmed cell death. Cross-talk between these pathways is mediated by BID, a proapoptotic BCL-2 family member. BID exists in the cytosolic fraction of living cells that becomes activated upon cleavage by caspase-8. The truncated cleavage product (tBID) then translocates to mitochondria and induces cytochrome c release. Interestingly, the functions of these caspases are inhibited by the inhibitor of apoptosis proteins (IAPs). The mitochondrial protein such as SMAC or DIABLO is released from mitochondria into the cytosol after apoptotic stimuli and promotes apoptosis by antagonizing IAPs.

14 days. Within 2 days of GnRH-A treatment, testicular concentrations of T declined markedly to 17.1% of control values and plasma T levels fell below detectable limits. Germ cell apoptosis, involving exclusively stages VII through VIII, was achieved by day 5. Within the study paradigm, the highest number of dying cells occurred by day 14, at which time a modest but significant increase in the incidence of apoptosis was also noted at stages other than VII through VIII. As shown in Figure 25-3, unlike our hyperthermia model, we found an increase in BAX and a decrease in BCL-2 expression in the mitochondrial fraction of testicular lysates after hormone withdrawal (Figure 25-3A). Such alteration in the BAX and BCL-2 ratio is accompanied by cytosolic translocation of mitochondrial cytochrome C and DIABLO (Figure 25-3B), activation of caspase-9 (Figure 25-3C) and caspase-3 (Figure 25-3D), and PARP cleavage (Figure 25-3E). These results indicate that withdrawal of gonadotropins and

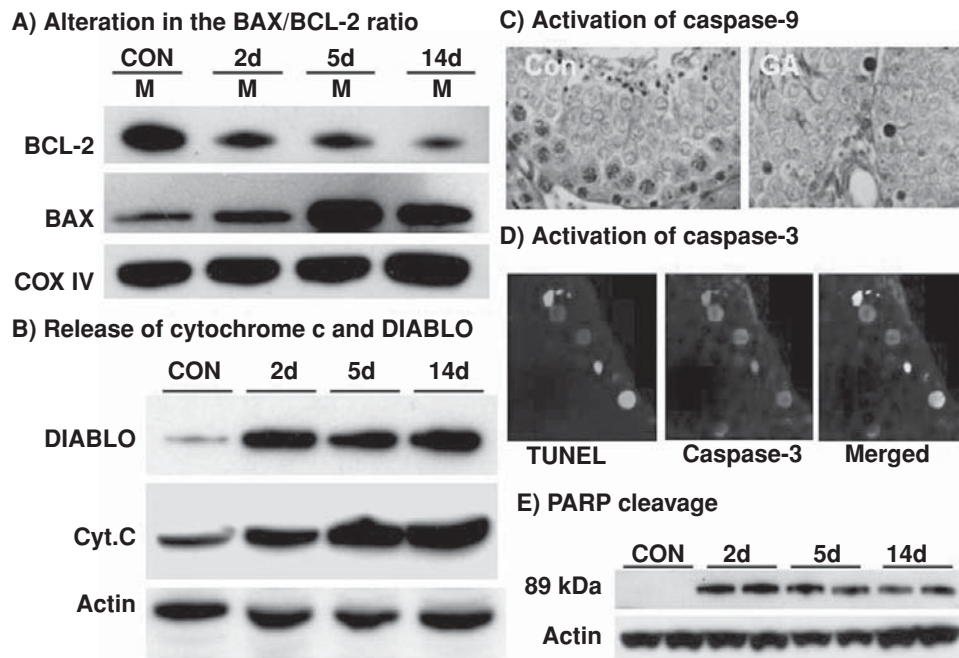


Figure 25-3. Hormonal deprivation results in activation of the intrinsic pathway signaling. (A) Western blot analysis shows an increase in BAX expression and a decrease in BCL-2 expression in the mitochondrial fraction (M) of testicular lysates after GnRH-A treatment. COX IV in the immunoblot is shown as a loading control. (B) Representative Western blots of cytosolic fractions of testicular lysates from control and rats 2, 5, and 14 days after GnRH-A treatment show a marked accumulation of DIABLO and cytochrome c in the cytosolic fractions after hormone deprivation. Actin in the immunoblot is shown as a loading control. (C) Portions of stage VII tubules from control and rats treated with GnRH-A for 5 days (GA) show activation of caspase-9 in selective germ cells after hormone deprivation, as detected by immunocytochemistry using an antibody that specifically detects active caspase-9. Scale bar, 15 μ m. (D) Confocal images of a portion of a stage VII tubule from a rat treated with GnRH-A for 5 days show TUNEL (green) and active caspase 3 (red) in germ cells. Scale bar, 15 μ m. (E) Caspase-3 activation after hormone withdrawal is associated with PARP cleavage, as evidenced by immunoblotting. This antibody recognizes only the cleaved PARP. From Vera et al., 2006. Reprinted with publisher permission. See Color Plate 24.

consequently intratesticular T induces germ cell apoptosis in the testis also by stimulating the intrinsic pathway signaling (Vera et al., 2006). Together, these results demonstrate that the mitochondria-dependent pathway appears to be the key apoptotic pathway for germ cell death in the testis.

4. THE FAS SIGNALING SYSTEM DOES NOT CONTRIBUTE TO HEAT- OR HORMONE DEPRIVATION-INDUCED MALE GERM CELL APOPTOSIS

To evaluate the involvement of the Fas signaling system in male germ cell apoptosis, in this study, we examined whether *gld* and *lpr^{cg}* mice, which harbor loss-of-function mutations in FasL and Fas, respectively (Nagata and Golstein, 1995), would confer resistance to heat-induced germ cell apoptosis. Similar to our rat model, scrota of *gld* and *lpr^{cg}* mice and their wild types (C57BL6J and MRL/Mpj, respectively) were exposed once to 22°C (control) or 43°C (heat-treated) for 15 minutes, and the

animals were killed at 0.5, 2, or 6 hours after heating. The incidence of germ cell apoptosis before and after heat treatment was similar in both wild-type and mutant mice, suggesting that germ cells from wild-type and mutant mice with loss-of-function mutations in Fas ligand and Fas, respectively, are equally sensitive to heat-induced apoptosis (Sinha Hikim et al., 2003a; Sinha Hikim et al., 2003b). Of note, the initiation of apoptosis was preceded by a redistribution of BAX from a cytoplasmic to perinuclear localization in heat-susceptible germ cells (Vera et al., 2004). The relocation of BAX is further accompanied by sequestration of ultra-condensed mitochondria into perinuclear areas of apoptotic germ cells and cytosolic translocation of mitochondrial cytochrome c and DIABLO and is associated with activation of the initiator caspase-9 and the executioner caspase-3 (Vera et al., 2004). Furthermore, we did not observe the presence of the truncated BID in either cytosolic or mitochondrial fractions of heat-treated testicular lysates of both wild-type and

mice lacking functional FAS, suggesting that the caspase-8-mediated cleavage of BID is not responsible for the observed release of cytochrome c from the mitochondria (Vera et al., 2004).

In additional studies, we further examined whether the FasL-defective gld mice would confer resistance to apoptosis induced by hormone withdrawal. We found that germ cells from wild-type and FasL-defective mice are equally sensitive to apoptosis triggered by hormone deprivation. These findings reinforce our earlier hypothesis that the intrinsic pathway signaling is the key apoptotic pathway for male germ cell death (Vera et al., 2006).

Nair and Shaha (2003) showed the involvement of the mitochondria-dependent pathway, characterized by loss of mitochondrial membrane potential, BAX translocation to mitochondria, cytochrome c release from mitochondria and subsequent activation of the caspase-9 and caspase-3, and PARP cleavage in diethylstilbestrol-induced testicular germ cell apoptosis in the rat. One other important finding that comes out of this study is the involvement of the FAS-FASL system, characterized by upregulation of FASL and FAS and activation of caspase-8 in germ cells. Theas and colleagues (2006) have further demonstrated the involvement of both death receptor and mitochondrial pathways in germ cell apoptosis in an experimental model of autoimmune orchitis. It would be interesting to know whether the link between these two pathways is the caspase-8-mediated cleavage of BID. Evidence exists that germ cells, in particular spermatocytes, are able to undergo tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis and that pretreatment with anti-DR5 antibody can increase their sensitivity to TRAIL-mediated apoptosis (McKee, Ye, and Richburg, 2006). Taken together, these results indicate that regulation of testicular germ cell apoptosis varies depending on the nature of apoptotic stimulus and can be triggered by more than one pathway.

5. P38 MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) AND NITRIC OXIDE (NO)-MEDIATED INTRINSIC PATHWAY SIGNALING CONSTITUTES A CRITICAL COMPONENT OF APOPTOTIC SIGNALING IN MALE GERM CELLS AFTER HORMONE DEPRIVATION

MAPKs comprise a family of serine/threonine kinases that function as critical mediators of a variety of extracellular signals. These kinases include the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK; also known as stress-activated protein kinase), and the p38 MAPK. To provide some insight into the upstream signaling pathways, we examined the role of

p38 MAPK and inducible nitric oxide synthase (iNOS) in apoptotic signaling of male germ cells in rats after hormone deprivation by a potent GnRH-A treatment. Activation of p38 MAPK, as evidenced by an increase in phospho-activating transcription factor-2 (ATF-2), was detected as early as 2 days after GnRH-A treatment and remained active thereafter throughout the treatment period (Figure 25-4A). Activation of p38 MAPK was also substantiated by immunohistochemistry and confocal microscopy. Compared with control, where no staining is detected, a strong phospho-p38 MAPK immunoreactivity was noted in the condensed nuclei of apoptotic germ cells after hormone withdrawal (Figure 25-4B, panels I–III). Co-staining for TUNEL and for phospho-p38 MAPK further confirmed activation of p38 MAPK only in those germ cells undergoing apoptosis (Fig. 25-4B, panels IV–VI). We found a similar profile in the induction of iNOS after GnRH-A treatment. Most importantly, p38 MAPK activation and iNOS induction within 2 days after GnRH-A treatment indicate that these events are indeed upstream of activation of apoptosis, which was first detected 5 days after GnRH-A treatment. p38 MAPK activation and iNOS induction were further accompanied by a marked perturbation of the BAX/BCL-2 rheostat, cytochrome c, and DIABLO release from mitochondria, caspase activation, and PARP cleavage (Vera et al., 2006). Concomitant administration of aminoguanidine (AG), a selective iNOS inhibitor, significantly prevented hormone deprivation-induced germ cell apoptosis (Vera et al., 2006). Relevant to this is the demonstration that such hormone deprivation-induced male germ cell apoptosis can be effectively prevented by minocycline (Castanares et al., 2005), which suppresses p38 MAPK activation, iNOS induction, and cytochrome c-mediated death pathway in other systems (Zhu et al., 2002; Teng et al., 2004; Wei et al., 2005). Induction of germ cell apoptosis after hormone withdrawal is independent of JNK or ERK (Castellanos, 2007).

6. P38 MAPK PATHWAY IS ALSO THE KEY PATHWAY FOR HEAT-INDUCED MALE GERM CELL APOPTOSIS

To characterize the upstream signaling pathways by which heat stress triggers male germ cell apoptosis, the contributions of the ERK, JNK, and p38 MAPK to stage-specific activation of germ cell apoptosis triggered by testicular hyperthermia were examined (Castellanos, 2007). Our data constitute the first demonstration that testicular hyperthermia results in stage- and cell-specific activation of both p38 MAPK and ERK, but not JNK. Activation of p38 MAPK, as evidenced by a significant ($P < 0.05$) increase (by 5.3-fold) in phospho-p38 MAPK

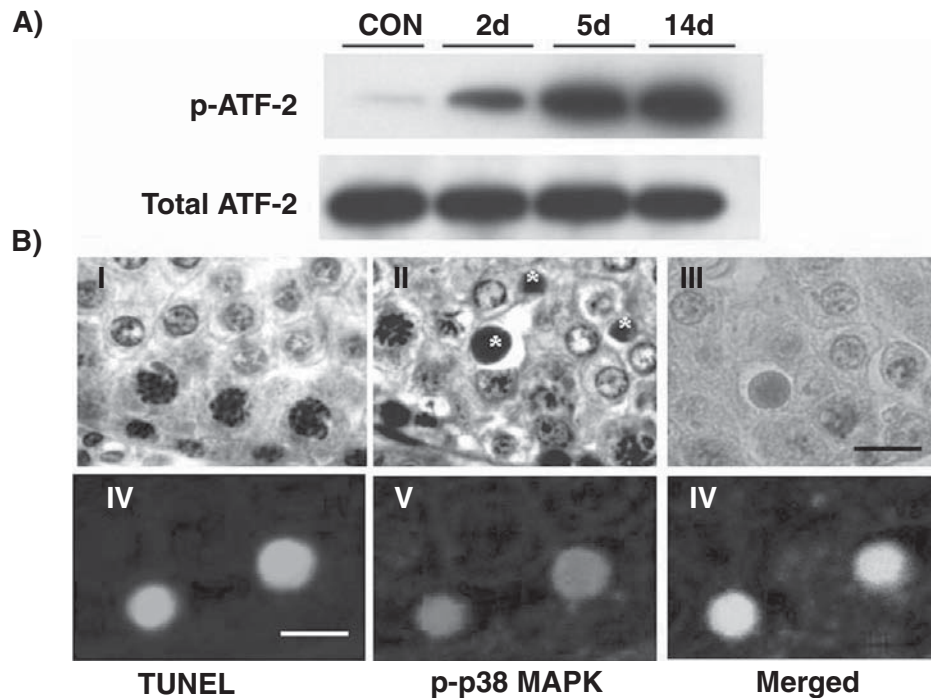


Figure 25-4. Activation of p38 MAPK in rat testes after GnRH-A treatment. (A) Analysis of p38 MAPK activation by Western blotting using phospho-ATF-2 (Thr 71) antibody in testicular lysates after GnRH-A treatment. Total ATF-2 in the immunoblot is shown as a loading control. (B) p38 MAPK activation visualized by immunocytochemistry and confocal microscopy. Portions of stage VII tubules from control (**panel I**) and rats treated with GnRH-A for 5 days (**panel II**) show a strong phospho-p38 MAPK immunoreactivity in the condensed nuclei of apoptotic germ cells (asterisk) after hormone withdrawal. A testicular section from a rat treated with GnRH-A for 5 days incubated with rabbit IgG (negative control) shows no such immunostaining in a stage VII tubule (**panel III**). Panels IV through VI, Confocal images show TUNEL (green), active p38 MAPK (red), and co-localization of TUNEL and active p38 MAPK (yellow) in apoptotic germ cells triggered by hormone deprivation. Scale bar, 15 μ m (**panels I–III**) and 10 μ m (**panels IV–VI**). From Vera et al., 2006. Reprinted with publisher permission. See Color Plate 25.

levels in testis lysates, was detected within one-half hour of heating and remained active thereafter throughout the treatment period. Because the phosphorylation status of BCL-2 plays an important role in its prosurvival activity (Halder, Basu, and Croce, 1998; Fan et al., 2000; Rajah, Lee, and Cohen, 2002; Bu et al., 2006), and this can be induced by p38 MAPK (Bu et al., 2006; Shimada et al., 2003), we next examined whether the increased germ cell apoptosis after heat stress is associated with BCL-2 phosphorylation. Compared with control, in which no staining was detected, we found marked increase in the serine-phosphorylated form of inactive BCL-2 only in heat-susceptible germ cells (Figure 25-5, panels I and II). Co-staining for TUNEL and phospho-BCL-2 further confirmed phosphorylation of BCL-2 only in those germ cells undergoing apoptosis (Figure 25-5, panels III through V). Most importantly, we further show that SB203580, a selective inhibitor of p38 MAPK, effectively suppressed BCL-2 phosphorylation and cytochrome c release and significantly ($P < 0.05$) prevented heat-induced germ cell apoptosis (Jia

et al., unpublished data). It is thus conceivable that the signal for activating mitochondria-dependent pathway during heat-induced male germ cell apoptosis emanates from p38 MAPK-mediated inactivation of BCL-2 through phosphorylation, thereby resulting in the perturbation of the BAX/BCL-2 rheostat in the mitochondria and the subsequent activation of the mitochondria-dependent death pathway.

Unlike p38 MAPK, we found activation of ERK within one-half hour of heating in the Sertoli cells at heat-susceptible stages (Figure 25-6). Thus the activation of ERK in the Sertoli cells is indeed upstream of activation of germ cell apoptosis, which was first detected 6 hours after heating (Yamamoto et al., 2001; Sinha Hikim et al., 2003b). Inhibition of ERK by U0126 had no effect on the incidence of heat-induced germ cell apoptosis, suggesting that ERK signaling may be dispensable for heat-induced germ cell apoptosis in the testis (Castellanos, 2007). At present, we do not know the possible significance of our findings. These observations, however, do suggest that not only germ cells, but also Sertoli cells

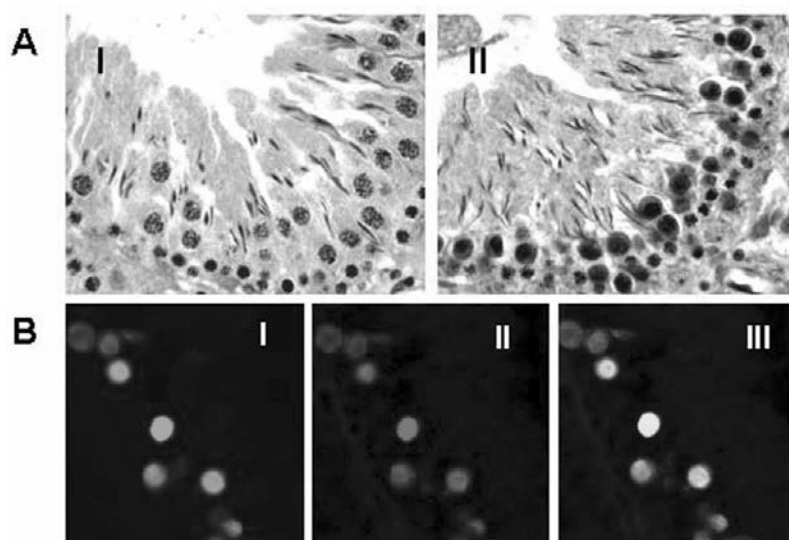


Figure 25-5. Testicular hyperthermia results in serine phosphorylation of BCL-2 in germ cells. (A) Portions of stage XII tubules from control (panel I) and a rat that had been exposed once to short-term local testicular heating (panel II) show serine phosphorylation of BCL-2 only in heat-susceptible late pachytene spermatocytes 6 hours after heating. Scale bar, 25 μ m. (B, panels I-III) Confocal images of late pachytene spermatocytes at stage XII from a heat-treated rat show TUNEL (green), phospho-BCL-2 (red), and colocalization of TUNEL and phospho-BCL-2 (yellow) in apoptotic germ cells 6 hours after heat treatment. Scale bar, 50 μ m. Reprinted from *The Journal of Steroid Biochemistry and Molecular Biology*, Hikim et al., Deciphering the pathways (2003), with permission from Elsevier. See Color Plate 26.

may be affected by heat treatment. In a recent study, we showed that heat treatment through activation of ERK induces dedifferentiation of adult Sertoli cells into immature states in monkeys (Zhang et al., 2006). Thus it is possible that the affected Sertoli cells could have compromised functions, which in turn sensitize these germ cells to apoptosis after heat stress.

Collectively, these data indicate that p38 MAPK-mediated signaling is also the key signaling pathway for heat-induced testicular germ cell apoptosis. However, unlike the hormone deprivation model, this

signaling pathway promotes germ cell apoptosis by provoking BCL-2 phosphorylation, leading to its inactivation, thereby resulting in the perturbation of the BAX/BCL-2 rheostat and the subsequent activation of the mitochondria-dependent death pathway.

7. CASPASE-2 IS AN UPSTREAM ACTIVATOR OF P38 MAPK AND NO-MEDIATED INTRINSIC PATHWAY SIGNALING

Of all caspases discovered to date, caspase-2 is the most evolutionarily conserved and plays an important role in inducing apoptosis in various cell systems. Caspase-2-mediated intrinsic pathway signaling has recently been implicated in the initial wave of germ cell apoptosis during the first round of spermatogenesis in mice (Zheng, Turner, and Lysiak, 2006). Lysiak and colleagues (2007) have further demonstrated the involvement of caspase-2-

mediated intrinsic pathway signaling in germ cell apoptosis in mice triggered by ischemia-reperfusion. To further explore the role of caspase-2, in a recent study, we sought to determine whether a specific inhibitor of caspase-2 (Z-VDAVDK-fmk) could prevent or attenuate heat-induced male germ cell apoptosis (Johnson et al., 2008). Quantitation of the TUNEL-positive germ cells revealed that Z-VDAVDK significantly ($P < 0.05$) prevented heat-induced germ cells apoptosis by 68.8%. Most notably, protection offered by the caspase-2 inhibitor occurred upstream of mitochondria,

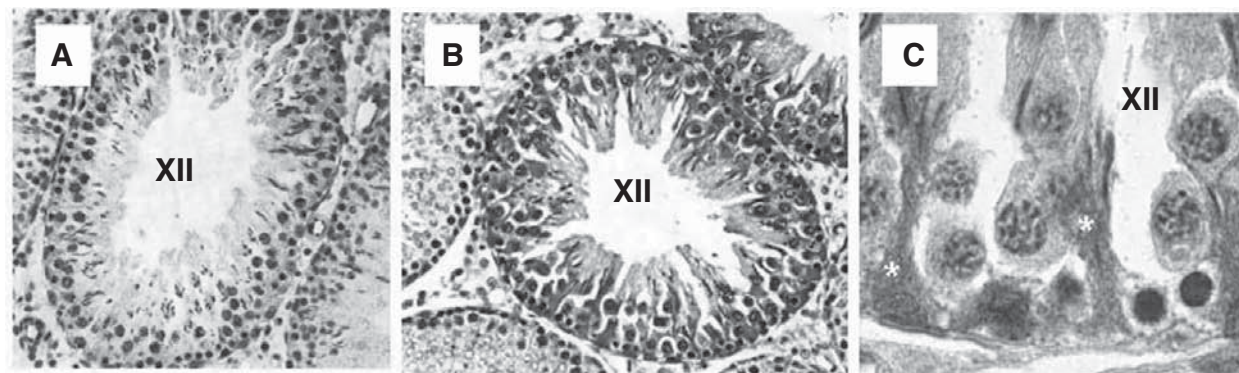


Figure 25-6. Activation of ERK in the Sertoli cells. Testicular sections from control (A) and rats that had been exposed once to short-term testicular heating (B and C) show activation of ERK at stage XII (a heat-sensitive stage) within one-half hour of heating. Scale bar, 50 μ m (A and B) and 15 μ m (C). See Color Plate 27.

involving suppression of p38 MAPK activation and iNOS induction and, in turn, suppression of the cytochrome c-mediated death pathway (Johnson et al., 2008). We found an almost identical level of protection (by 67.0%) of testicular germ cells from heat-induced apoptosis in mice pretreated with a Quinoline-Val-asp (Ome)-CH₂-O-ph (Q-VD-OPH), a broad-spectrum pan caspase inhibitor (Vera et al., 2005). However, compared with Z-VDAVDK, the protection offered by Q-VD-OPH was independent of mitochondrial cytochrome c release and occurred by inhibiting caspase activation (Vera et al., 2005). Together, these studies indicate that caspase-2 activation is needed to fuel cytochrome c or DIABLO release from mitochondria.

8. SIGNALING PATHWAYS FOR TESTICULAR GERM CELL DEATH IN NONHUMAN PRIMATES

Our group has also characterized the signaling pathways in inducing accelerated apoptosis after testicular hyperthermia, hormonal deprivation, or combined interventions in a nonhuman primate model (Lue et al., 2006). Treatment with T, heat, or both in adult cynomolgus monkeys led to sustained activation of both ERK and p38 MAPK. Activation of both these kinases were accompanied by an increase in BCL-2 levels in both cytosolic and mitochondrial fractions of testicular lysates (BAX levels remained unaffected) and cytochrome c and DIABLO release from mitochondria. These treatments also resulted in inactivation of BCL-2 through phosphorylation at serine 70, thereby favoring the death pathway. We conclude that the serine phosphorylation of BCL-2 and activation of the p38 MAPK-mediated mitochondria-dependent pathway are critical for male germ cell death in monkeys (Jia et al., 2007).

9. SIGNALING PATHWAYS FOR TESTICULAR GERM CELL DEATH IN HUMAN

Having established that p38 MAPK-mediated intrinsic pathway signaling constitutes a critical component of apoptotic signaling in male germ cells in rats (Vera et al., 2006) and monkeys (Jia et al., 2007), we next evaluated the efficacy of iNOS as well as p38 MAPK inhibitors in preventing or attenuating human male germ cell apoptosis induced by deprivation of survival factors. As expected, culturing seminiferous tubules for 4 hours resulted in clear apoptotic DNA laddering, as detected by Southern blot analysis of DNA fragmentation. Concomitant treatments with SB 203580, a p38 MAPK inhibitor, and AG, a selective iNOS inhibitor, significantly suppressed low molecular DNA

fragmentation induced by culturing segments of human seminiferous tubules under hormone-free conditions. We further examined the induction of iNOS during human male germ cell apoptosis by immunoblotting from tubular samples cultured under hormone-free conditions. No iNOS expression was detected in the noncultured seminiferous tubule fragments. In contrast, culturing seminiferous tubules for 4 hours resulted in induction of iNOS and that could be effectively suppressed by SB 203580 treatment, indicating that p38 MAPK is an upstream activator of iNOS during human male germ cell apoptosis.

Together, these results establish a new signal transduction pathway involving p38 MAPK and iNOS that, through activation of the intrinsic pathway signaling, promotes male germ cell death in response to a lack of hormonal stimulation across species (Vera et al., 2006; Jia et al., 2007).

10. COMPLETE REVERSIBILITY OF SPERMATOGENESIS AFTER DISCONTINUATION OF SUPPRESSION OF GONADOTROPINS BY EXPERIMENTAL CONTRACEPTIVES

Increased germ cell apoptosis plays an important role in organized regression of spermatogenesis after hormonal suppression, including human (Wang et al., 2007). With this hormonal suppression, azoospermia (no sperm in ejaculate) or severe oligozoospermia (< 3 million sperm per mL of semen) sufficient for contraceptive purposes can be achieved (Wang and Swerdloff, 2004). Thus it is important to investigate the reversibility of spermatogenesis after cessation of hormonal contraceptive treatment. In a recent study, we undertook an integrated analysis of data from 1,549 participants in hormonal contraceptive studies (T with or without progestagen) in 20 centers across the globe (Liu et al., 2006). The data show full reversibility within a predictable time course (Liu et al., 2006). This finding provides a strong foundation on which a safe, reliable, and reversible contraception based on hormonal suppression of spermatogenesis could soon become available.

11. CONCLUSIONS AND PERSPECTIVES

It is now widely accepted that male germ cell apoptosis is a genetically driven form of cell death and is a critical prerequisite for functional spermatogenesis. There is increasing evidence that null mutations of a number of genes in mice result in severe spermatogenic disruption and infertility through accelerated germ cell apoptosis. Most notably, it appears that null mutation of some genes, expressed in many tissues, including the testis,

can have specific effects on germ cell apoptosis and spermatogenesis. Detailed characterization of the underlying mechanism of those defects in these mutant mice will provide insight into the basic control mechanism of male germ apoptosis.

Recently, tissue-specific *in vivo* RNA interference (RNAi) approach has been used to elucidate the cell type-specific function of Williams' tumor 1 (WT1) in regulating spermatogenesis (Rao et al., 2006). Mice depleted of WT1 in Sertoli cells exhibit increased germ cell apoptosis, loss of adherence junctions, and impaired fertility. By substituting different lineage-specific or regulated promoters and stem-loops corresponding to different gene targets, this system has the potential to knock down the expression of virtually any gene in a cell-type specific and temporally regulated manner. This novel *in vivo* RNAi approach may avoid the frequently observed problems of early lethality or developmental redundancy.

Our understanding of the regulation of male germ cell apoptosis has greatly expanded in recent years. Much progress has been made toward unraveling the key signal transduction pathways in apoptotic signaling of murine and primate male germ cells. However, significant gaps remain in our knowledge base. Emerging evidence now suggests a more direct role of cellular metabolism in governing cell death through either activation of a specific death pathway or loss of a critical survival pathway. During spermatogenesis, Sertoli-germ cell metabolic cooperation is essential for germ cell survival (Boussouar and Benahmed, 2004). Systemic glucose is taken by Sertoli cells, processed glycolytically into lactate, and transported across the plasma membrane to the germ cells by specific monocarboxylate transporter. The challenge is how to characterize the metabolic networks, using stable isotope-based metabolic flux phenotyping in conjunction with gas chromatography and mass spectrometry (Lee, 2006), and the novel aspects of those networks that actually necessary for male germ cell death. Metabolic profiling and its integration with signal transduction pathways inducing germ cell death will provide insight into how perturbation of the metabolic cooperation between Sertoli and germ cells affect germ cell survival.

Future efforts toward improved fertility control and clinical management of infertility associated with reduced sperm production in men are hampered by incomplete understanding of the processes responsible for normal germ cell homeostasis. Elucidation of the metabolic and molecular mechanisms by which various environmental stresses and male contraceptive approaches regulates germ cell death will fill a major gap in our knowledge of this fundamental biological process.

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1. INTRODUCTION

Cardiovascular disease is the most common cause of death in the world. Regulated forms of cell death play critical roles in cardiovascular disease. In particular, apoptosis and necrosis, and perhaps autophagic cell death, are causal components in the pathogenesis of the most common and lethal cardiovascular syndromes: myocardial infarction and heart failure. This chapter summarizes the mechanisms and physiologic impact of regulated cell death in the cardiovascular system.

2. CELL DEATH IN THE VASCULATURE

2.1. Apoptosis in the developing blood vessels

Vascular development requires not only the formation, but also the regression, of blood vessels. For example, components of the first, second, and fifth aortic arches involute during embryonic development. Similarly, the ductus arteriosus, which shunts blood past the lungs in fetal life, becomes a fibrotic vestigial structure after the initiation of postnatal pulmonary function. Changes of this sort are accompanied by apoptosis of endothelial and smooth muscle cells,^{1,2,3,4} suggesting that cell death is involved in vascular regression and remodeling.

A causal connection between cell death and vascular remodeling has been demonstrated by genetic manipulations in the mouse. For example, loss of survival pathways can cause marked reductions in blood vessel abundance. This is illustrated by endothelial cell-specific deletion of IKK β (I κ B [inhibitor of κ B] kinase β), which results in caspase activation, marked reductions in liver blood vessels, and lethality at embryonic days 13.5 through 15.5.⁵ Similarly, knockout of Bcl-2 (B-cell leukemia/lymphoma-2) results in apoptosis, reduc-

tion in the abundance of endothelial cells and pericytes, and decreased retinal artery density in postnatal mice.⁶ Conversely, loss of apoptosis signaling can lead to extra vessels. This is illustrated by combined knockouts of Bax (Bcl-2-associated X protein) and Bak (Bcl-2 homologous antagonist/killer), which display loss of normally occurring endothelial cell apoptosis with persistence of fetal retinal vessels.⁷

A variety of physiologic stimuli, including shear stress, interactions with extracellular matrix, and soluble factors, such as vascular endothelial growth factor, promote endothelial cell survival.⁸ Conversely, endothelial cells in regressing capillary beds can be killed by Wnts secreted from macrophages.⁹ Moreover, reductions in capillary flow resulting from the killed endothelial cells can then reduce shear stress and delivery of nutrients, thereby leading to further endothelial cell death.^{10,11}

In contrast, the role of apoptosis in the remodeling of larger vessels is not known. For example, reduction of carotid blood flow in adult rabbits and mice stimulates endothelial cell and/or smooth muscle cell apoptosis. The vascular lumen becomes smaller, but this may be due to reactive smooth muscle cell proliferation, matrix deposition, and overall vessel shrinkage. Moreover, apoptosis of vascular cells in arteries may cause only variable and, in some cases, transient vascular changes.^{12,13} For these reasons, the significance of flow-mediated cell death in the remodeling of large vessels remains unclear.

2.2. Apoptosis in atherosclerosis

The advanced human atherosclerotic plaque is formed through a complex series of events that involve all arterial cell types (Figure 26-1), as follows: (1) Endothelial cell dysfunction/damage is an initiating event. (2) This

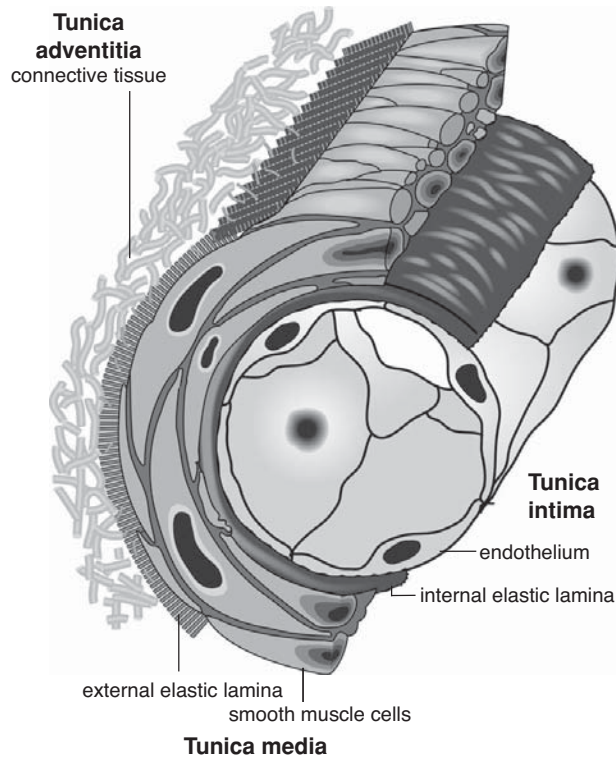


Figure 26-1. Normal human artery consists of three layers. The tunica intima (intima), the layer closest to the lumen of the vessel through which blood flows, is composed of a single layer of endothelial cells resting on a basement membrane. The tunica media (media) is comprised of multiple layers of vascular smooth muscle cells. The tunica adventitia (adventitia), the outermost layer, is composed of fibroblasts and collagen-rich matrix containing nerves, lymphatics, and small blood vessels. Internal and external elastic laminae separate intima from media and media from adventitia, respectively. Reprinted with permission from School of Anatomy and Human Biology – The University of Western Australia. See Color Plate 28.

leads to recruitment of monocytes/macrophages into the intima. (3) Uptake of lipids into the macrophages results in their transformation to foam cells. (4) Foam and endothelial cells signal the migration of vascular smooth muscle cells from media to intima, where their replication and collagen/matrix production form a fibrous cap. This fibrous cap separates the thrombogenic, lipid-rich “necrotic core” of the plaque from the flowing blood.^{14,15} Myocardial infarction (“heart attack,” discussed later in this chapter), is the acute death of heart muscle cells resulting from the sudden cessation of blood flow in a coronary artery. Rather than being precipitated by progressive arterial narrowing, most myocardial infarctions are triggered by acute rupture of the fibrous cap of the plaque (Figure 26-2).^{16,17} Contact between thrombogenic factors in the plaque and the flowing blood then activates platelets, leading to subsequent thrombosis and coronary artery occlusion.

Levels of apoptosis are low to undetectable in the normal vessel wall¹⁸ but increase progressively during plaque development.^{18,19,20,21} This cell death occurs in both the necrotic core and the fibrous cap. Most apoptosis in plaques involves vascular smooth muscle cells and macrophages. We focus first on smooth muscle cell death.

2.2.1. Vascular smooth muscle cells

Transgenic mice that express diphtheria toxin receptor exclusively in arterial smooth muscle cells have been used to investigate a causal connection between smooth muscle cell apoptosis and atherogenesis (Figure 26-3a). These studies show that modestly elevated levels of vascular smooth muscle cell apoptosis (0.8%–1.1%) – comparable to those seen in human plaques – are sufficient to accelerate plaque progression in an atherogenic milieu (*apolipoprotein E*^{−/−} [*apoE*^{−/−}] mice on a high-fat diet).²² The underlying mechanisms are incompletely understood but involve proinflammatory effects of apoptotic cells.²³

Studies have also linked vascular smooth muscle cell apoptosis with plaque rupture (Figure 26-3b). Increased levels of vascular smooth muscle cell apoptosis are associated with rupture-prone coronary artery plaques^{24,25} in patients with unstable angina as compared with those with stable angina.²⁶ The most direct evidence, however,

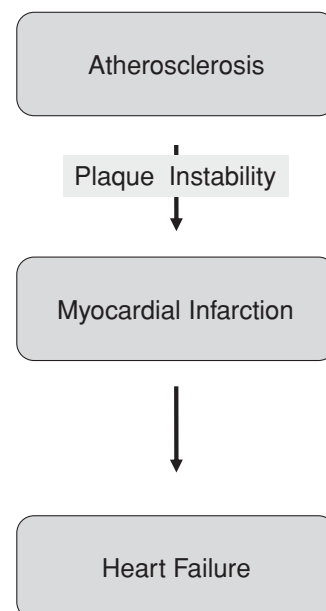


Figure 26-2. Relationship between atherosclerosis, myocardial infarction, and heart failure. Rupture of an atherosclerotic plaque acutely precipitates myocardial infarction. Myocardial infarction can lead to heart failure. See text for details.

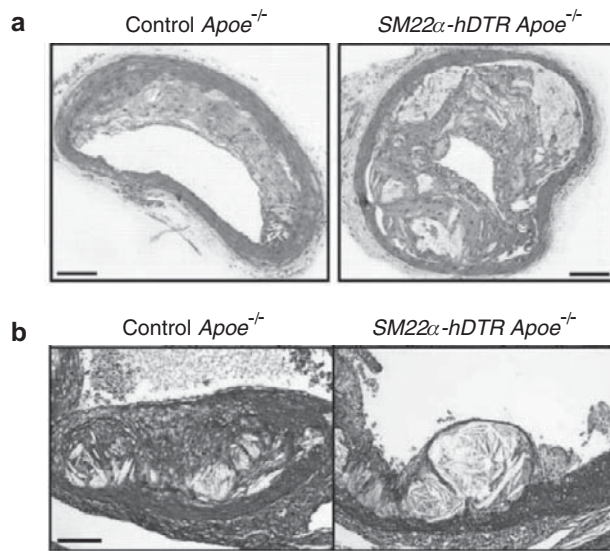


Figure 26-3. Vascular smooth muscle cell apoptosis accelerates atherosclerotic plaque progression²² and induces plaque vulnerability.²⁷ Transgenic mice were created in which expression of the human diphtheria toxin receptor was targeted to arterial smooth muscle cells. Animals were crossed onto an *apoe*^{-/-} background and fed a high-fat diet to induce atherosclerosis. Apoptosis of arterial smooth muscle cells was induced by administration of diphtheria toxin. This model was used to study the effects of arterial smooth muscle cell apoptosis on plaque progression and instability. Apoptosis accelerated plaque formation in the brachiocephalic artery as shown by increased plaque area by hematoxylin and eosin staining (**a**). Plaque vulnerability was increased by apoptosis (not shown) in the carotid artery, as illustrated by thinning of the fibrous cap, loss of collagen and matrix, increased necrotic core size, cellular debris, and inflammation (Masson trichrome staining in **b** and not shown). Space bars 100 μ M (**a**) and 50 μ M (**b**). (**a**) Reproduced with permission from Clarke MC, Littlewood TD, Figg N, Maguire JJ, Davenport AP, Goddard M, Bennett MR. Chronic apoptosis of vascular smooth muscle cells accelerates atherosclerosis and promotes calcification and medial degeneration. *Circ Res*. 2008;102:1529–38. (**b**) Reprinted by permission from Macmillan Publishers Ltd: Clark MC, Figg N, Maguire JJ, Davenport AP, Goddard M, Littlewood TD, Bennett MR. Apoptosis of vascular smooth muscle cells induces features of plaque vulnerability in atherosclerosis. *Nat Med*. 2006;12:1075–80. See Color Plate 29.

is provided by the diphtheria toxin receptor transgenic mouse, described in the previous paragraph. Induction of vascular smooth muscle cell apoptosis results in thinning of the fibrous cap, loss of collagen and matrix, accumulation of lipids and cellular debris within an increased necrotic core, and formation of inflammatory foci within the atherosclerotic lesion.²⁷ Although thinning of the fibrous cap does not progress to overt rupture in this model, these data suggest that vascular smooth muscle cell apoptosis can precipitate features of plaque instability in an atherosclerotic context. Although these studies demonstrate the sufficiency of vascular smooth muscle cell apoptosis for plaque instability, the necessity of cell death in this process remains to be evaluated.

2.2.2. Macrophages

Macrophages are the most frequent apoptotic cell type in advanced lesions.¹⁸ Apoptosis of these cells may have varying effects on atherosclerosis at different times in the disease process. During atherogenesis, macrophage apoptosis appears to reduce lesion formation. Consistent with this, diphtheria toxin-mediated killing of macrophages in *apoe*^{-/-} mice fed a high-fat diet results in reduced plaque and necrotic core size.²⁸ Conversely, adoptive transfer of *bax*^{-/-} bone marrow into mice lacking the low-density-lipoprotein receptor and on a high-fat diet showed increased plaque area compared with mice reconstituted with wild-type cells.²⁹ These studies suggest that macrophage apoptosis limits plaque development. In contrast, macrophage apoptosis in established plaques may increase the necrotic core size without affecting plaque size.³⁰

A related theory is that changes in clearance of apoptotic bodies promote progression of established plaque. Inhibition of phagocytosis, through loss-of-function mutations in Mertk (MER tyrosine kinase) or lactadherin, accelerates atherosclerosis in established plaques and is accompanied by increases in necrotic core size.^{31,32,33} In fact, the efficiency of phagocytosis appears decreased in the milieu of atherosclerotic plaques.³⁴ The precise relationship between apoptosis and phagocytosis with regard to atherogenesis remains to be delineated.

Endoplasmic reticulum (ER) stress has also been implicated in the role of macrophages in atherosclerosis. ER stress may be stimulated by lipid-mediated oxidative damage and/or increased accumulation of free cholesterol within macrophages.^{35,36} Markers of the unfolded protein response (UPR) are activated during all phases of plaque development.³⁷ Deletion of CHOP [C/EBP (CCAAT/enhancer binding protein)-homologous protein], which transcriptionally activates genes that mediate both UPR and ER stress-induced apoptosis, lowers rates of macrophage apoptosis and reduces necrotic core size in atherosclerosis-prone mice.³⁸ Furthermore, CHOP and GRP78 (glucose-regulated protein 78, another UPR marker), are increased in ruptured, but not stable, human plaques. These observations suggest a role for ER stress in necrotic core formation and human plaque rupture.³⁹

2.2.3. Regulation of apoptosis in atherosclerosis

Resident vessel wall cells are resistant to apoptosis induced by death ligands, in part because of increased levels of FLIP [FLICE (FADD-Like IL-1 β -converting

enzyme)-inhibitory protein] and cellular inhibitor of apoptosis protein 1 (c-IAP1) and decreased levels of FADD (Fas-associated via death domain), Fas ligand, and caspases. Death receptors are also sequestered within these cells.⁴⁰ Indeed, death ligand expression by endothelial cells can induce apoptosis in invading inflammatory cells as a protective mechanism.^{41,42} Endothelial cells and vascular smooth muscle cells, however, can be primed to undergo death ligand-induced apoptosis by cytokines or stimuli that traffic death receptors to the cell surface, such as interferon- γ ,⁴³ nitric oxide,⁴⁴ and p53⁴⁵ or that downregulate protective genes such as inhibitors of apoptosis (IAPs).⁴⁶ Atherosclerosis itself increases the sensitivity of both endothelial cells and vascular smooth muscle cells to undergo apoptosis. Both macrophages and cytotoxic T cells^{47,48,49} can induce apoptosis in these cells.^{44,50} In addition, plaque vascular smooth muscle cells show increased sensitivity to apoptosis compared with their normal counterparts.²¹ Increased sensitivity can occur via overexpression/activation of p53,⁵¹ with subsequent reduction in survival signaling such as that mediated by insulin-like growth factor 1 (IGF-1). For example, oxidative stress (e.g., as it occurs within an atherosclerotic plaque) can induce post-translational modification of p53 that renders it competent to repress IGF-1 receptor (IGFR) transcription.⁵² In addition, IGF-1 signaling can also be dampened by oversecretion of IGF-1 binding proteins from plaque vascular smooth muscle cells.⁵³

2.2.4. Necrosis and autophagy in atherosclerosis

Little is known about the role of nonapoptotic forms of cell death in atherosclerosis. There is evidence that cultured macrophages, vascular smooth muscle cells, and endothelial cells can undergo necrosis and/or autophagic cell death in response to experimental stimuli.^{54,55,56,57} It remains unclear, however, whether these forms of cell death take place in intact atherosclerotic plaques and if so, contribute to the pathogenesis of atherosclerosis.

3. CELL DEATH IN THE MYOCARDIUM

Myocardial infarction and heart failure are the most common and lethal heart syndromes. Death of cardiac myocytes is a critical component in the pathogenesis of both, although the quantities and kinetics of cell death in each differ greatly.

The defining feature of myocardial infarction is the acute death of large numbers of cardiac myocytes during a relatively short interval (hours to <1 day). The

precipitant is ischemia, defined as decreased blood flow in one of the coronary arteries, the vessels that feed the heart muscle itself. Ischemia is precipitated by the rupture of an atherosclerotic plaque in the coronary artery (Figure 26-2). Typically, before rupture, atherosclerotic plaques cause only minor to moderate obstruction of the coronary artery. After rupture, the recruitment of platelets leads to rapid and sustained thrombotic occlusion. The consequences of myocardial infarction can be acute (arrhythmias, sudden death, structural damage to the heart) and long term (arrhythmias, sudden death, and heart failure).

Heart failure can most easily be thought of as a “weakening” of the heart muscle such that the strength of contraction is inadequate to propel blood to meet the metabolic needs of the body. There are many underlying causes of heart failure. These include hypertension, valvular heart disease, congenital heart disease, congenital cardiomyopathies, and toxins. Among the most common, however, is the occurrence of prior myocardial infarction(s) (Figure 26-2). Myocardial infarction elicits heart failure through two pathophysiologic mechanisms: (1) acute loss of functioning heart muscle from the infarction itself (*infarct zone*), and (2) long-term, pathological post-infarct remodeling in regions of the heart that were not involved in the myocardial infarction (*noninfarcted myocardium*). Remodeling of the noninfarcted myocardium is triggered by a complex array of mechanical, humoral, and neural pathways that sense the loss of functioning heart muscle in the infarct zone. Cardiac myocytes exhibit activation of a variety of stress pathways, leading initially to myocyte widening and thickening of heart chamber walls. At advanced stages, however, myocytes elongate, walls become thin, cardiac chambers dilate, and contractile function deteriorates. Cardiac myocyte death often ensues during this progression. These remodeling changes characterize not only the failing noninfarcted myocardium after myocardial infarction, but also the myocardium in advanced heart failure of any etiology.

In contrast to the robust, relatively short-lived burst of cell death during myocardial infarction, the magnitude of cardiac myocyte death in the failing heart is only modestly elevated (discussed in Section 3.2). However, cells can continue to die over the protracted course of heart failure, which may be months to several years in humans. This can result in significant cumulative loss of cells over the course of the disease. In the discussion that follows, we will consider the various death programs that operate during myocardial infarction and heart failure and the causal effects of cell death on cardiac function.

3.1. Cell death in myocardial infarction

The most significant type of myocardial infarction is ST-segment elevation myocardial infarction (the term is based on its electrocardiographic features). *Infarction* itself refers to cell death in a tissue, regardless of the underlying death process. ST-segment elevation myocardial infarction results from the acute thrombotic occlusion of a coronary artery, the immediate consequence of which is ischemia that eventually leads to cell death in the segment of myocardium supplied by the artery. The prompt re-establishment of adequate blood flow in the coronary artery limits myocardial damage.^{58,59,60,61} Accordingly, optimal therapy is acute reperfusion, currently best accomplished through angioplasty/stenting.

Ischemia subjects the myocardium to deficits of oxygen, nutrients, and survival factors and to surpluses of waste products resulting in intracellular acidosis. Although the overall benefit of reperfusion in saving myocardium is firmly established, reperfusion itself may trigger a set of detrimental processes collectively termed *reperfusion injury*.⁵⁹ While somewhat controversial, these include rapid re-activation of the electron transport chain and generation of reactive oxygen species, mitochondrial and cytoplasmic Ca^{2+} overload, overly rapid resolution of intracellular acidosis that can paradoxically trigger opening of the mitochondrial permeability transition pore (MPTP; discussed later in this chapter), and inflammation.⁵⁹ Current work is directed toward reducing these potential toxicities to further enhance the benefit of reperfusion.

Human myocardial infarction can be modeled in animals by permanent surgical occlusion of the left coronary artery or by an extended period of left coronary artery occlusion followed by reperfusion, known as ischemia-reperfusion. These models simulate the clinical courses of untreated myocardial infarction and myocardial infarction treated with reperfusion, respectively.

Permanent coronary occlusion and ischemia-reperfusion both result in large levels of apoptotic,^{62,63} necrotic,^{63,64,65,66} and possibly autophagic^{67,68} cell death. The creation of a definitive temporal and spatial map of the magnitudes and kinetics of each type of cell death in these models has been hampered by differences in markers and methodologies used in the various studies. Nevertheless, it can be stated that apoptosis, even as measured by late markers such as terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) or DNA laddering, is evident within a few hours of permanent coronary artery occlusion and

even sooner after ischemia-reperfusion.⁶⁹ Necrosis, as evaluated by electron microscopy and histology, occurs within hours of permanent coronary occlusion.^{63,70} Autophagy (not autophagic cell death), as evaluated by LC3 (microtubule-associated protein 1 light chain 3) conversion or dots, can be detected less than 1 hour into reperfusion after ischemia.⁶⁷

In addition to timing, location is also an important consideration. In a brain undergoing stroke, ischemia induces necrosis in the central infarct zone and apoptosis in surrounding penumbra.⁷¹ In contrast, the infarcting heart does not exhibit a grossly demarcated penumbra. Moreover, the infarct zone in the heart appears to involve both apoptosis and necrosis, although the relative magnitudes of each and their contributions to infarct size and cardiac dysfunction are poorly understood. The peri-infarct zone (the region immediately surrounding the infarct) and noninfarcted myocardium also undergo apoptosis after myocardial infarction with the magnitude decreasing progressively with the distance from the infarct zone.^{72,73} The extent to which necrosis takes place in these zones has not been precisely defined.

As will be discussed later, the deaths of cardiac myocytes play critical roles in the structural and functional changes in the heart during and after myocardial infarction. It should be noted, however, that other myocardial cell types (fibroblasts, endothelial cells, smooth muscle cells, neural cells, and others) also die at significant rates during infarction.^{70,74} In fact, myocytes may actually be relatively resistant to apoptosis because of low Apaf-1 (apoptotic protease activating factor-1) levels that may increase X-linked IAP-mediated inhibition of apoptosis in these cells.^{75,76} It is possible that the loss of these non-myocytes, which has not been studied in detail, also affects cardiac damage and dysfunction during infarction.

3.1.1. Apoptosis in myocardial infarction

The discussion here focuses on experimental perturbations of the central apoptosis pathways in intact animals that have revealed causal connections between cardiac myocyte apoptosis and subsequent infarct size and cardiac function. As described, these studies show that apoptosis plays a critical role in the genesis of a myocardial infarction and the resulting cardiac dysfunction. In the whole-animal studies cited below, cardiac myocyte apoptosis is often measured by TUNEL (in conjunction with immunostaining for a cardiac myocyte-specific marker) and infarction (total cell death) with 2,3,5-triphenyl tetrazolium chloride (a substrate for mitochondrial reductases).

Mice deficient in Fas (*lpr* [*lymphoproliferation*] on MRL/MpJ background, used before the onset of autoimmune manifestations) exhibit 64% less cardiac myocyte apoptosis and 62% smaller infarcts after ischemia-reperfusion in vivo compared with wild-type mice.⁷⁷ Similar reductions in apoptosis are observed after ex vivo ischemia-reperfusion of isolated, buffer-perfused hearts obtained from *lpr* mice. Moreover, the reperfusion phase of ischemia-reperfusion induced the secretion of Fas ligand and tumor necrosis factor- α (TNF- α) from isolated, buffer-perfused hearts, suggesting a paracrine loop.⁷⁸ These experiments indicate a role for Fas-mediated apoptosis in ischemia-reperfusion. The recent recognition that death receptors can also participate in necrosis signaling^{79,80} raises the possibility that the rescue in infarct size in *lpr* mice may represent decreases in both apoptosis and necrosis.

The influence of TNF- α signaling on myocardial infarction is more complex because of effects on cardiac myocyte viability (survival, apoptosis, necrosis), contractility, and inflammation. An added intricacy relates to the existence of two receptors, TNF- α receptor subtypes 1 and 2 (TNFR1 and TNFR2). Deletion of either TNFR1 or TNFR2 does not alter infarct size after permanent coronary occlusion in vivo or ischemia-reperfusion in isolated, buffer-perfused hearts.^{81,82} However, concomitant deletion of both receptors increases infarct size 39% and 40%, respectively, in these models. Conversely, low-level cardiac myocyte-specific transgenic expression of TNF- α or TRAF2 (TNF receptor-associated factor 2) on a wild-type background decreases infarct size and improves function after ischemia-reperfusion in isolated, buffer-perfused hearts.⁸² These data suggest that both TNFR1 and TNFR2 transduce survival signals in the context of prolonged myocardial ischemia with or without reperfusion. Paradoxically, other studies report that pharmacological antagonism of TNF- α , which would be predicted to decrease signaling through both TNF receptors, reduces infarct size.^{83,84,85} Moreover, in some models of heart failure (as opposed to myocardial infarction), TNFR1 appears to transduce pathogenic signals, whereas TNFR2 is protective.^{86,87} The reasons for these discrepant findings are unclear but may reflect differences in models or the pleiotropic effects of TNF signaling in the heart.

Given the great importance of nutrients/energy for normal cardiac function, it is not surprising that the intrinsic apoptosis pathway also plays an important role in cardiac myocyte apoptosis and myocardial infarction. Deletion of the multidomain proapoptotic protein Bax results in a 35% reduction in infarct size after permanent coronary occlusion in vivo⁸⁸ and 49% reduction in

isolated, buffer-perfused hearts subjected to ischemia-reperfusion.⁸⁹ Decreases in cardiac myocyte apoptosis and cardiac dysfunction parallel these reductions in infarct size. Similarly, mice lacking the multidomain proapoptotic protein Bak exhibit 36% smaller infarcts after ischemia-reperfusion in vivo (Whelan, Jha, and Kitsis, unpublished data). Thus, in distinction to some cell culture models that reveal a high degree of redundancy between Bax and Bak,⁹⁰ full cardiac myocyte killing during infarction in vivo appears dependent on both Bax and Bak. The importance of the intrinsic pathway in myocardial ischemia-reperfusion is confirmed by transgenic over-expression of the antiapoptotic protein Bcl-2 in the heart. Infarct size after ischemia-reperfusion in vivo is reduced 64%⁹¹ and 48%⁹² in two independent mouse lines.

The actions of the various multidomain Bcl-2 proteins are mediated through a variety of mechanisms. These include their effects on mitochondrial apoptogen release, ER Ca²⁺ handling,^{93,94} and possibly the unfolded protein response.⁹⁵ Which of these mechanisms predominate with respect to their effects on myocardial infarction is not clear.

A multitude of BH3 [B cell leukemia/lymphoma-2 (Bcl-2) homology domain 3]-only proteins activate Bax and Bak directly⁹⁶ or indirectly.⁹⁷ Deletion of the BH3-only protein Bid [BH3-interacting domain death agonist], which links extrinsic and intrinsic pathways, decreases infarct size by 53% after ischemia-reperfusion in vivo.⁹⁸ Notably, Bid activation during ischemia-reperfusion may reflect cleavage by calpains as well as caspase-8.⁹⁹ Loss of p53-upregulated modulator of apoptosis (PUMA) also decreases infarct size by 50% in isolated, buffer-perfused hearts subjected to ischemia-reperfusion.¹⁰⁰

It is clear from the preceding discussion that both the extrinsic and intrinsic pathways mediate cardiac myocyte death during myocardial infarction. Although most apoptosis inhibitors target either the intrinsic (e.g., Bcl-2) or extrinsic (e.g., FLIP) pathways, ARC (apoptosis repressor with a CARD [caspase recruitment domain]) antagonizes both pathways through inhibition of death-inducing signaling complex (DISC) formation and Bax activation and by promoting p53 nuclear hyperexport.^{101,102} The effects of ARC deletion on infarct size are unresolved, as one group observed an increase in infarct size that was not seen in an independently generated null mouse (Donath et al.¹⁰³; Ji, Jha, and Kitsis, unpublished data). The reasons for this discrepancy are unclear but may include genetic compensation and technical variation in the experiments. In addition, differences between ARC knockout and

wild-type animals may be further blunted because of dramatic reperfusion-induced decreases in ARC protein levels resulting from degradation in the ubiquitin-proteasomal pathway.^{104,105} Consistent with this, even low-level (three-fold) cardiac-specific transgenic over-expression of ARC is adequate to reduce infarct size by approximately 40% after ischemia-reperfusion in vivo.¹⁰⁶

The intrinsic pathway is also modulated at the postmitochondrial level. IAPs bind and inhibit already activated effector caspases¹⁰⁷ and may interact with procaspase-9 to interfere with apoptosome assembly.¹⁰⁸ In addition, IAPs can ubiquitinate effector caspases, targeting them for degradation in the proteasome.^{109,110} Cardiac-specific transgenic over-expression (two-fold) of cIAP2 reduces infarct size by 32% after ischemia-reperfusion in vivo.¹¹¹ Although caspase-3/7 activity was not measured in this study, TUNEL staining was shown to be decreased. Apart from their actions in the postmitochondrial apoptosis pathway, however, cIAP1 and cIAP2 also inhibit necrosis and apoptosis pathways induced by death receptor signals. These effects are mediated by non-canonical K63-polyubiquitination of receptor interacting protein 1 (RIP1) by cIAP1 or cIAP2 (in conjunction with TRAF2), leading ultimately to NF- κ B (nuclear factor κ light-chain enhancer of activated B cells) activation and transcription of survival genes.^{112,113,114,115,116,117} In light of this complexity, the antiapoptotic/necrotic mechanisms that mediate reduction of infarct size by cIAP2 remain undefined. The pleiotropic actions of the IAPs, however, suggest that they may be useful in novel therapies to limit infarct size.

Smac/DIABLO (second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI) and Omi/HtrA2 (Omi/High temperature requirement protein A2) are mitochondrial apoptogens that neutralize IAPs through direct binding, resulting in the displacement of active caspases.^{118,119} In addition, Omi/HtrA2 degrades IAPs irreversibly through its serine protease activity.¹²⁰ UCF-101 [(5-[5-(2-nitrophenyl)furfuryliodine]-1,3-diphenyl-2-thiobarbituric acid)] is a small-molecule inhibitor of the Omi/HtrA2 serine protease activity. UCF-101 reduces infarct size by 47%¹²¹ and 30%¹²² after ischemia-reperfusion in vivo in mice and rats, respectively. This was accompanied by attenuation of IAP loss and decreases in caspase activity, apoptosis, and cardiac dysfunction. The efficacy of UCF-101 is notable in light of potential redundancy from Smac/DIABLO. A possible explanation may relate to the fact that UCF-101 antagonizes IAP degradation, an irreversible event that can be carried out by Omi/HtrA2 but not Smac/DIABLO. Another possible explanation for the efficacy of UCF-101 is that, by maintaining IAP levels, it

promotes the death receptor survival pathway, discussed previously. Despite these open mechanistic questions, there is significant interest in the clinical potential of this compound.

Polycaspase inhibitors have been shown to decrease infarct size by 21% to 52% in mice, rats, and rabbits after ischemia-reperfusion in vivo.^{123,124,125,126} The mechanisms responsible for these effects are incompletely understood, however. For example, it is unclear whether amelioration of cardiac damage is mostly due to antagonism of upstream or downstream caspases. In a similar vein, it is not known whether downstream caspase inhibition lessens mitochondrial dysfunction or mitochondrial necrotic events.¹²⁷

Another level of complexity involves several striated muscle-specific caspase-3 substrates. The contractile proteins α -cardiac actin and troponin T are cleaved by caspase-3 in cardiac myocytes, and experimental cleavage of α -cardiac actin decreases contractile function in skinned fibers.¹²⁸ Although these caspase-3 cleavage events may simply be part of the cell death pathway, they raise the possibility that caspases can also cause contractile dysfunction apart from inducing cell death.

3.1.2. Necrosis in myocardial infarction

The extensive studies in the preceding section establish that cardiac myocyte apoptosis is important in ischemia-reperfusion-induced myocardial cell death; however, myocardial infarction has traditionally been thought of as involving predominantly necrosis. Although necrosis is often envisioned as an unregulated processes, work over the past decade in worms and mice has demonstrated that at least some instances are highly controlled. This regulation involves two pathways, one mediated by cell surface death receptors^{79,80,116,117} and the other by events at the inner mitochondrial membrane^{65,66,129,130,131,132,133}. Most investigations involving myocardial ischemia-reperfusion have focused on the mitochondrial necrosis pathway.

In this context, Ca^{2+} overload and oxidative stress trigger opening of the MPTP, a channel in the inner mitochondrial membrane.¹²⁹ Ischemia leads to intracellular acidosis, causing increases in intracellular Na^+ via the Na^+/H^+ exchanger. Increases in intracellular Ca^{2+} subsequently result via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and through Ca^{2+} -induced Ca^{2+} release from the ER/sarcoplasmic reticulum. Meanwhile, reperfusion induces oxidative stress and rapid alkalization, both resulting in MPTP opening.¹³⁴ MPTP opening has two major consequences: (1) loss of inner mitochondrial membrane potential resulting in cessation of

adenosine diphosphate→adenosine triphosphate synthesis and (2) influx of water down its osmotic gradient into the matrix, resulting in massive mitochondrial swelling and even rupture of the outer mitochondrial membrane.¹²⁹ The resulting release of apoptogens through a Bax/Bak-independent mechanism may then engage the downstream apoptotic machinery and further augment necrotic killing.

The biochemical composition of MPTP is not known. It is clear, however, that the peptidyl-prolyl *cis-trans* isomerase cyclophilin D in the mitochondrial matrix regulates MPTP.¹²⁹ Deletion of *ppif* (encoding cyclophilin D) in the mouse confers marked resistance to necrosis without affecting apoptosis.^{65,66,131} In two independent knockout mice, myocardial infarct size was reduced 40% and 75% after ischemia-reperfusion *in vivo*.^{65,66} These data show that necrosis is an important death program during ischemia-reperfusion and that the mitochondrial pathway plays a critical role.

The role of the death receptor necrosis pathway in myocardial ischemia-reperfusion is less defined. However, administration of necrostatin-1, a small-molecule inhibitor of the kinase activity of RIP1,¹³⁵ reduces infarct size 40% to 67% after ischemia-reperfusion *in vivo*.^{136,137} Thus the death receptor necrosis pathway also appears to be involved. Interestingly, necrostatin-1 had no additional effect in the setting of cyclophilin D deletion, suggesting that the death receptor and mitochondrial necrosis pathways intersect. Although reactive oxygen species resulting from catabolic pathways activated by RIP3¹³⁸ may provide a link between these two pathways, our current understanding of these connections is rudimentary at the mechanistic level.

3.1.3. Autophagy in myocardial infarction

Macroautophagy helps cells survive periods of starvation and stress in organisms ranging from yeast to humans.¹³⁹ In a number of systems, the question has been raised regarding whether autophagy, under some circumstances, can beget cell death. In fact, there may be evidence to this effect in some systems.¹⁴⁰ However, the testing of this hypothesis has suffered because of (1) a conceptual gap regarding what death machinery carries out autophagic cell death; (2) an unclear understanding of what trigger converts a survival process to a death process; and (3) most importantly, the absence of markers for autophagic cell death as opposed to autophagy itself. Despite these limitations, there are several cardiac studies that deserve consideration.

Autophagy is induced during both permanent coronary occlusion and ischemia-perfusion *in vivo*, but

with important differences in mechanism and significance.^{67,68} 5' Adenosine monophosphate-activated protein kinase (AMPK), an inducer of autophagy, is activated during permanent coronary occlusion, but not during ischemia-reperfusion. On the other hand, the abundance of Beclin-1, also a mediator of autophagy, increases during ischemia-reperfusion. Transgenic over-expression of a dominant negative AMPK during permanent coronary occlusion *in vivo* inhibits induction of autophagy and results in increased infarct size.⁶⁸ These data suggest that autophagy plays a protective role in persistent myocardial ischemia without reperfusion, which is consistent with its role in starvation. Of note, however, potential effects of AMPK on apoptosis and metabolism may cloud this interpretation. This result contrasts with what is observed during ischemia-reperfusion. Deletion of a single Beclin-1 allele decreases autophagy associated with decreased infarct size after ischemia-reperfusion *in vivo*.⁶⁷ This suggests that, in contrast to the protective role of autophagy in permanent coronary occlusion, autophagy appears to play a pathogenic role in ischemia-reperfusion.

3.2. Cell death in heart failure

In the failing heart, the myocardium is too weak to pump blood efficiently to meet the metabolic needs of the various tissues. Heart failure is a final common outcome for many serious cardiac diseases. A common etiology is prior myocardial infarction(s), which acutely destroy segments of functional myocardium (as discussed previously) and cause the remaining noninfarcted myocardium to fail gradually. Additional causes include hypertension, valvular heart disease, and cardiomyopathies, among others. In each of these cases, heart failure develops gradually – over months to several years – such that the final phenotype cannot be distinguished by etiology.

Heart failure is complex at the physiologic, cellular, and molecular levels.¹⁴¹ There are multiple abnormalities in cell signaling (including activation of a variety of stress pathways), Ca²⁺ handling and excitation-contraction coupling, cytoskeleton, contractile proteins, energetics, and extracellular matrix. Many of these processes culminate in cell death. However, it is important to keep in mind that heart failure may also ensue because of cardiac myocyte dysfunction without cell death. Thus cell death is an important factor in heart failure, but not the only one.

Although the sequence of events in the development of heart failure varies with the underlying etiology, the process often starts with a mechanical “load” being

imposed on the heart. For example, in hypertension, the left ventricle (major pumping chamber) is subjected to increased pressure. It responds by undergoing hypertrophic growth, in which the walls of ventricle becomes thicker as a result of the individual cardiac myocytes becoming larger (but not more numerous). After a sustained period of pressure overload, the heart transitions from hypertrophy to failure. Microscopically, this is characterized by cardiac myocytes becoming elongated rather than wider. Macroscopically, the left ventricular chamber becomes larger in volume and the thick, hypertrophic walls become thinned. Functionally, the contractions of the heart weaken. Symptoms of heart failure develop, including tiredness, shortness of breath especially on exertion, and swelling of the legs and feet. This is a lethal disease, with patients dying either from “pump failure” or arrhythmias (sudden cardiac death).

As noted previously, myocardial infarction is characterized by a large burst of cardiac myocyte apoptosis that lasts only for hours. In contrast, the frequency of cardiac myocyte apoptosis in the failing heart is very low (0.08%–0.25%), but still ~10- to 100-fold higher than that in normal controls (0.001%–0.002%).^{142,143,144} The frequencies of necrosis and autophagic cell death are not known with any degree of reliability. We will review studies that assess whether these death programs play a causal role in heart failure.

3.2.1. Apoptosis in heart failure

Can rates of cardiac myocyte apoptosis as low as 0.08% to 0.25% (0.001%–0.002% in controls) contribute significantly to human heart failure^{142,143,144}? To address this issue experimentally, transgenic mice were created with cardiac-specific expression of a human caspase-8 allele^{145,146} that exhibits low levels of constitutive activation.¹⁴⁷ These mice develop lethal heart failure at 3 to 9 weeks of age (Figure 26-4). In contrast, mice expressing equivalent levels of a similar, but enzymatically dead, caspase-8 allele had normal hearts. Mice with heart failure exhibited rates of cardiac myocyte apoptosis of 0.023%, 15-fold higher than those of controls (0.016%). These apoptotic rates of 0.023%, however, are 4 to 10 times *lower* than those in patients with heart failure, suggesting that a low (but relatively elevated) level of cardiac myocyte apoptosis is sufficient to cause, over time, lethal heart failure. To test the dependency of heart failure on caspase activation and apoptosis in this model, a polycaspase inhibitor was administered before the development of heart failure. Caspase inhibition markedly attenuated development of abnormalities of cardiac structure and function. These data indicate that the modest rates

of cardiac myocyte apoptosis observed in heart failure can contribute to the pathogenesis of this syndrome.

Signals from several cell surface receptors (type 1 angiotensin II receptor, α 1-adrenergic receptor, endothelin receptor) modulate cardiac hypertrophy and failure via G α q. Transgenic expression of G α q bypasses the need for these receptors in the induction of cardiac hypertrophy and failure.¹⁴⁸ Analysis of hearts from G α q transgenic mice revealed increases in transcripts encoding Nix/Bnip3L (Nip3 [19-kD interacting protein-3]-like protein X/Bcl-2/adenovirus E1B 19 kD interacting protein 3-like), a BH3-only-like Bcl-2 protein. Expression of Nix/Bnip3L in transgenic mice was sufficient to cause extensive cardiac myocyte apoptosis and lethality.¹⁴⁹ A subset of G α q female mice developed overwhelming heart failure with pregnancy.¹⁵⁰ Cardiac myocyte apoptosis, cardiac dysfunction, and mortality is attenuated in these mice by caspase inhibition or concurrent transgenic expression of sNix, a dominant negative splice variant of Nix/Bnip3L.¹⁵¹ These data link pathways that mediate cardiac myocyte hypertrophy and apoptosis and demonstrate a role for apoptosis in heart failure.

The preceding two studies used models in which apoptosis had been induced to demonstrate that inhibition of apoptosis could improve heart failure. The next study tests whether apoptosis inhibition will ameliorate heart failure induced by clinically relevant pathophysiologic stresses. Ischemia-reperfusion was used to cause myocardial infarction, which, as previously noted, can precipitate failure of the noninfarcted myocardium. Bnip3 (Bcl-2/adenovirus E1B 19 kD interacting protein 3) is a BH3-only-like protein in the same subfamily as Nix/Bnip3L (discussed previously). Of note, Bnip3 is induced in cardiac myocytes by hypoxia. Deletion of Bnip3 in the mouse does not affect infarct size after ischemia-reperfusion *in vivo* – possibly because of inadequate time for its induction during infarction. In contrast, absence of Bnip3 significantly reduces cardiac myocyte apoptosis in the peri-infarct zone and noninfarcted myocardium and attenuates pathological myocardial remodeling. These data demonstrate that cardiac myocyte apoptosis is a causal component of postinfarct heart failure.

In addition to the induction of apoptotic mediators, heart failure may also be triggered by the loss of survival mechanisms. One such survival pathway is mediated by gp130, a subunit of the receptors that bind several prosurvival cytokines of the interleukin-6 family, some of which also induce cardiac hypertrophy. Cardiac-specific deletion of gp130 in the mouse results in no baseline abnormalities. The imposition of

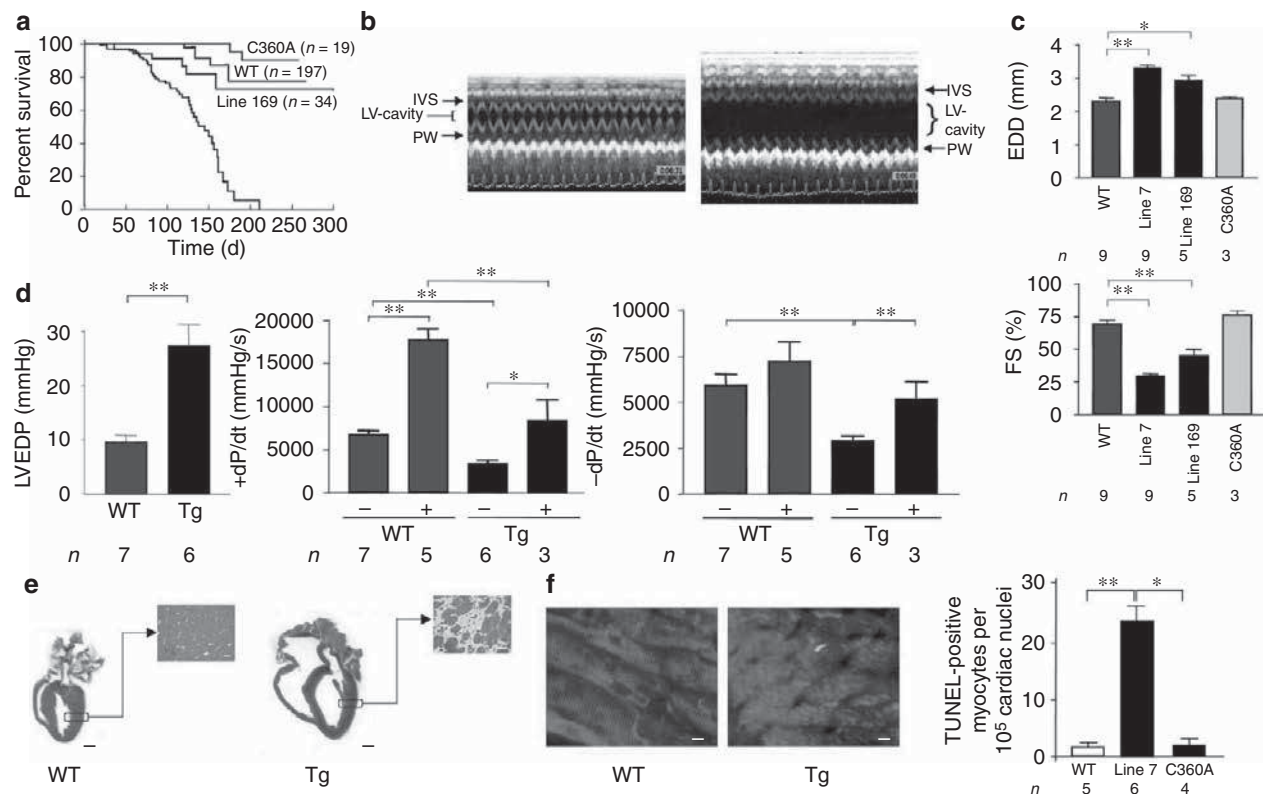


Figure 26-4. Modest, but elevated, rates of cardiac myocyte apoptosis are sufficient over time to induce lethal heart failure.¹⁴⁷ Transgenic mice were generated with cardiac-specific expression of a human caspase-8 allele that exhibits low constitutive activity (line 7 higher expressors, line 169 lower expressors). This transgene protein consists of the p20 and p10 subunits of procaspase-8 fused to a trimer of mutated FK binding protein (FKBP) and a myristoylation signal for plasma membrane targeting. Unexpectedly, even in the absence of a dimeric FKBP ligand, modest – but abnormal – rates of cardiac myocyte apoptosis ensue: 0.023% in transgenic mice (line 7) versus ~0.016% in either wild-type (WT) mice or transgenic mice expressing a catalytically inactive version (C360A) of the same transgene (f). This low level of cardiac myocyte apoptosis is sufficient to cause heart failure between 3 and 9 weeks of age (b–e) and death between 2 and 7 months of age (a). (b, c) Echocardiography with quantification. WT (left) and line 7 transgenic (right). LV, left ventricle; IVS, inter-ventricular septum; PW, left ventricular posterior wall; EDD, left ventricular end-diastolic dimension; FS, left ventricular fractional shortening. (d) Hemodynamics. LVEDP, left ventricular end-diastolic pressure; + and –, dP/dt maximal rate of rise or fall of left ventricular pressure. (e) Hematoxylin and eosin staining of hearts and Masson trichrome staining of tissues in insets. Space bar 1 mm (hearts) and 25 μ m (insets). Reprinted with permission from Wencker D, Chandra M, Nguyen KT, Miao W, Garantziotis S, Factor SM, Shirani J, Armstrong RC, Kitsis RN. A mechanistic role for cardiac myocyte apoptosis in heart failure. *J Clin Invest*. 2003;111:1497–504. See Color Plate 30.

hemodynamic overload from aortic constriction, however, precipitates extensive cardiac myocyte apoptosis and severe heart failure.¹⁵² The physiologic role of gp130 appears to be phosphorylation of STAT3 (signal transducer and activator of transcription 3) during times of stress, which in turn activates transcription of Bcl-x_L.

3.2.2. Necrosis in heart failure

Rates of necrosis are increased in human heart failure,^{142,153} but the significance of necrotic death in the pathogenesis of this syndrome is not known. Diastolic Ca²⁺ overload is a characteristic of heart failure, and

increased mitochondrial Ca²⁺ can trigger MPTP opening and necrosis. Accordingly, to assess the pathogenic significance of necrosis in heart failure, transgenic mice were generated with cardiac-specific, inducible over-expression of the β 2a subunit of the L-type Ca²⁺ channel.¹⁵⁴ Ca²⁺ overload-induced necrosis in the hearts of these mice elicited heart failure, which was rescued by deletion of cyclophilin D but not by over-expression of Bcl-2. Cyclophilin D absence also ameliorated heart failure in a model of doxorubicin-induced cardiomyopathy. Thus, in addition to the previously discussed role of apoptosis in heart failure, these studies suggest that cardiac myocyte necrosis is also a pathogenic component.

3.2.3. Autophagy in heart failure

Rates of autophagy-related cell death in cardiac myocytes have been reported to be increased in human heart failure,^{153,155,156} although the methodology used to assess this process is not conclusive. Moreover, the role of autophagy and autophagy-related death in heart failure remains controversial.

At least in some situations, autophagy plays a protective role in the heart. This is illustrated by the massive induction of heart failure that results from inhibition of autophagy using cardiac myocyte-specific deletion of *atg5* in the first week of postnatal life.¹⁵⁷ In contrast, when *atg5* is deleted at embryonic day 8, no abnormalities are evident at birth – possibly because of compensation. However, imposition of aortic constriction precipitates heart failure more readily in the *atg5* null, than in wild-type mice. This study demonstrates a critical role for autophagy in maintaining cardiac structure and function at baseline and during stress.

Conflicting results were obtained when the role of autophagy in aortic constriction-induced heart failure was studied using *beclin-1*^{+/-} mice to inhibit autophagy.¹⁵⁸ When subjected to aortic constriction, *beclin-1*^{+/-} mice exhibit less heart failure compared with wild-type mice. Conversely, over-expression of *beclin-1* enhances aortic constriction-induced heart failure. Thus, in this study, autophagy appears to play a pathogenic role in heart failure. The explanation for the apparent discrepancy in the results obtained with *atg5*^{-/-} and *beclin-1*^{+/-} mice, each studied in an aortic constriction model, are not readily apparent, but may relate to differences in the manipulated genes and the apparently more severe aortic constriction achieved in the *beclin-1*^{+/-} experiments. Further experimentation will be needed to resolve these issues.

The *beclin-1*^{+/-} mice have also been used to assess the role of autophagy in another form of heart failure, desmin-related cardiomyopathy.¹⁵⁹ This is an inherited disease that involves aggregation of desmin and α B crystallin and can be modeled in mice by transgenic over-expression of the R120G mutant of α B crystallin in cardiac myocytes. Inhibition of autophagy in this model exacerbates the accumulation of polyubiquitinated proteins, severity of heart failure, and mortality. Thus autophagy plays a protective role in this model of desmin-related cardiomyopathy. The two *beclin-1*^{+/-} studies that have been discussed show that autophagy may function as a protective or a detrimental adaptation depending on the stimulus. Specifically, in a cardiac proteotoxicity model, autophagy is essential for mitigating heart failure. In contrast, in a hypertrophy

model (with secondary mechanical and metabolic changes, among many others), autophagy may contribute to the adverse effects on cardiac structure and function. An important challenge is to understand these divergent effects at the mechanistic level.

In summary, the studies in this section have considered the relationship between autophagy and heart failure. Conflicting results have been obtained in some cases, which may be model- or gene-related. As importantly, it should be emphasized that these studies involve the role of autophagy. Autophagic cell death, if it exists, was not directly measured. This is largely because, in contrast to autophagy, markers are lacking for autophagic cell death. A better understanding of how autophagy impacts on the multiple complex events of heart failure (e.g., contractile proteins, mitochondria, energetics) may provide insights into its role in this syndrome.

4. CONCLUDING REMARKS

We have presented studies that demonstrate that various regulated forms of cell death play roles in the pathogenesis of cardiovascular disease. In the vasculature, apoptosis of vascular cells appears to be involved in atherogenesis and perhaps plaque instability. In the myocardium, regulated cardiac myocyte death is a causal component in the pathogenesis of myocardial infarction and heart failure. At least apoptosis and necrosis appear to be involved. One important challenge is to determine the mechanistic relationships between these death programs.

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1. INTRODUCTION TO MUSCLE

Skeletal muscle constitutes ~40% of total body weight, and its primary function is to provide force and energy for locomotion, breathing, and postural support. It can also act as a source of heat production during cold-induced stress or exercise. Skeletal muscle is a highly adaptable tissue that exhibits a remarkable range of plasticity in response to different stimuli. It possesses distinctive structural and biochemical properties that distinguish it from all other tissues. There is a significant variation in size, type, and shape of skeletal muscles within the body. However, the underlying morphological structure remains the same. Common to all skeletal muscle is the presence of multiple myonuclei per cell, the innervation of several cells by single α -motor neurons, and the presence of ligaments and tendons.

As illustrated in [Figure 27-1](#), numerous muscle fasciculi bundled together constitute the skeletal muscle. The muscle fasciculi are formed by a multitude of tightly bound individual muscle cells, or myofibers. Each myofiber consists of multiple myofibrils, which can be further subdivided into individual segments known as sarcomeres, the basic contractile unit of the muscle. Sarcomeres consist of thick (myosin) and thin (actin) filaments, arranged in a specific hexagonal ratio. Six actin filaments surround each myosin chain, and three myosin thick filaments border each actin molecule. A sarcomere is defined as the space between two neighboring Z-lines. The I-band, composed mainly of actin filaments, encompasses the Z-line and shortens during muscle contraction as the actin filaments slide over the myosin heavy chain. The A-band corresponds to the width of the myosin filaments and it remains static during muscle contraction. Sarcomeres arranged end to end

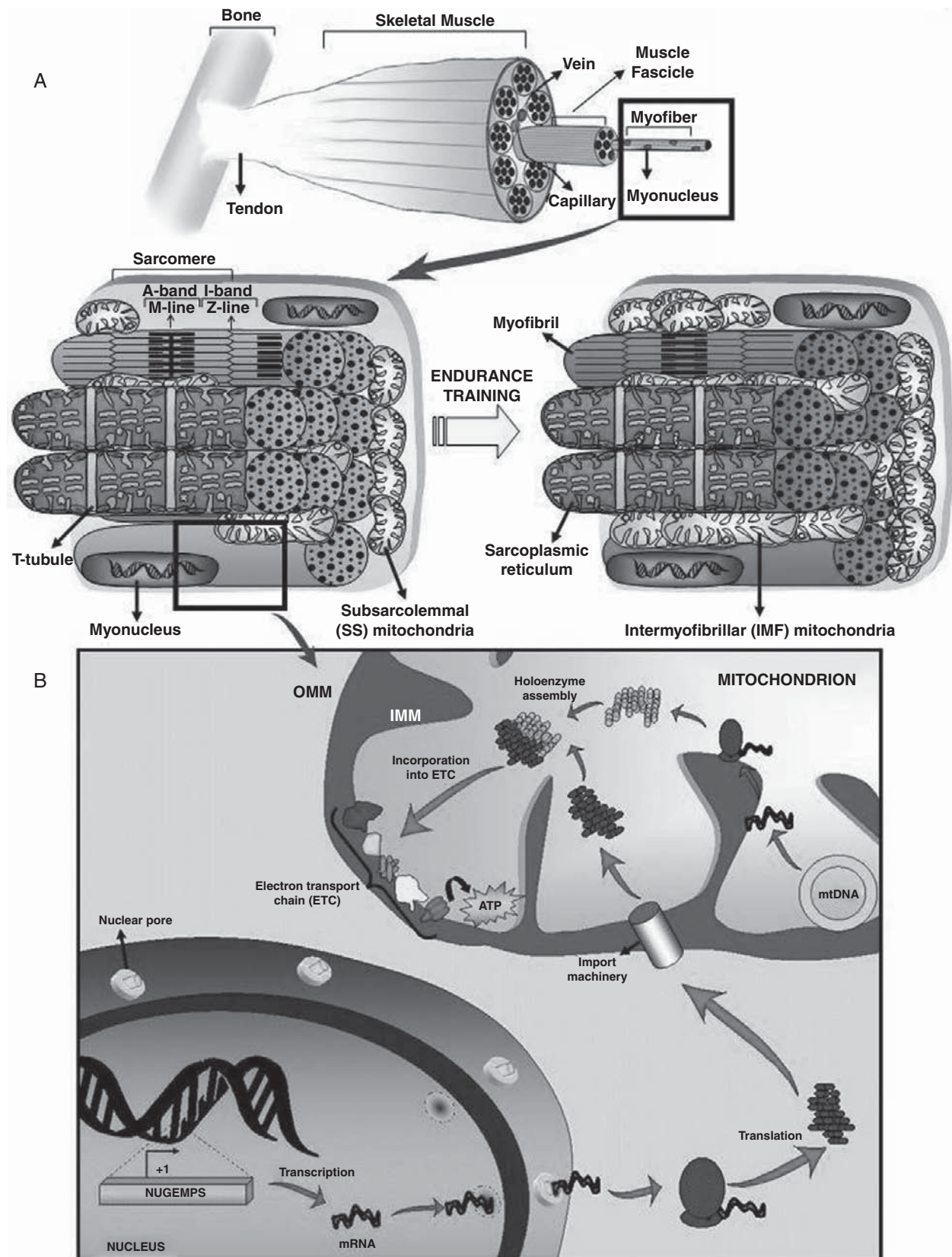
within each myofibril give skeletal muscle its unique striated appearance in electron micrographs.

Within each myofiber, the sarcoplasmic reticulum (SR) exists as a membranous network that surrounds every myofibril ([Figure 27-1](#)). The SR functions as a calcium (Ca^{2+}) reservoir in skeletal muscle. During muscle contraction, an action potential travels down the invaginations of the plasma membrane known as t-tubules and induces Ca^{2+} release from the sarcoplasmic reticulum. Once released, Ca^{2+} binds to troponin and promotes a shift in the position of the inhibitory protein tropomyosin, which masks the myosin binding sites on the actin fibers. This allows for actin and myosin interaction and, consequently, muscle contraction as actin slides over the myosin molecules and the sarcomeres shorten in length.

Another important characteristic of skeletal muscle is that it houses two discrete pools of mitochondria ([Figure 27-1](#)). Mitochondria located beneath the sarcolemma of the muscle fiber are known as subsarcolemmal (SS) mitochondria, whereas those interspersed in between the myofibrils are called intermyofibrillar (IMF) mitochondria. The former comprise approximately 20% of the total mitochondrial volume and are mainly responsible for generating energy for membrane-bound functions. Conversely, IMF mitochondria account for the remaining 80% of the mitochondrial content and are presumably responsible for generating ATP for contractile purposes.

1.1. Skeletal muscle adaptation to endurance training

Skeletal muscle is a highly pliable tissue that can adapt phenotypically and metabolically to a range of



functional demands. One example of this adaptation is in response to repeated bouts of endurance exercise. This is referred to as endurance training, and it can involve modalities such as wheel or treadmill running or it can be imposed via chronic electrical stimulation. These paradigms induce changes in muscle oxidative phenotype and result in improved endurance performance. The transition toward a more oxidative phenotype with endurance training includes increases in expression of myoglobin, an enhanced rate of angiogenesis, a shift from type II toward type I myosin heavy chain expression, and an increase in mitochondrial biogenesis. A coordinated interplay between the nuclear and mitochondrial DNA regulates the process of mitochondrial biogenesis (Figure 27-1). Exercise stimulates signaling pathways that upregulate the transcription of several regulatory proteins. Peroxisome proliferator activated receptor γ coactivator-1 α (PGC-1 α) has been referred to as the “master regulator” of mitochondrial biogenesis because of its role in the specific activation of nuclear genes encoding mitochondrial proteins. PGC-1 α can bind to and activate the transcription of the nuclear respirator factor-1 (NRF-1) and NRF-2, two transcription factors that are responsible for expressing mitochondrial oxidative genes such as cytochrome c; proteins of the electron transport chain (ETC); mitochondrial import proteins; mitochondrial transcription factors Tfam, TFB1M, and TFB2M; and heme biosynthesis proteins. The mitochondrial-destined proteins interface with the protein import machinery that spans the outer and inner mitochondrial membranes to enter the mitochondria. In contrast to the nuclear genome, mito-

chondrial DNA (mtDNA) encodes only 13 proteins of the ETC, but the expression of each of these proteins is crucial for proper mitochondrial assembly and function. Tfam, TFB1M, and TFB2M regulate the transcription and translation of mtDNA-derived proteins, which act singularly, or in combination, with nuclear-encoded proteins to form multi-subunit complexes that are incorporated into the ETC.

1.2. Myonuclear domains

In addition to adaptations induced by endurance training, skeletal muscle can also respond to other physiologic perturbations, such as resistance exercise, muscle disuse, and injury/disease. In the case of a functional overload such as weight training, muscle hypertrophy is manifest. In contrast, muscle disuse is characterized by myofiber atrophy, whereas in response to injury, myofibers can repair themselves and regenerate. The growth and regenerative capacity of muscle is primarily mediated through muscle satellite cells. These cells occupy hollow dimples in the muscle fiber between the basal lamina and sarcolemma, and they are found in all vertebrates. Satellite cells are mononucleated progenitor cells that can be activated by exercise or injury-induced signals to give rise to myogenic precursor cells or myoblasts. During embryonic development, conditions of muscle regeneration, or muscle growth, satellite cells multiply, fuse, and donate their nuclei to the myofiber to induce muscle hypertrophy (Figure 27-2). Signals that mediate the fusion of myoblasts are largely dependent on the nuclear factor of

Figure 27-1 (facing page). Unique morphology of skeletal muscle and exercise-induced mitochondrial biogenesis. **A.** Skeletal muscle cells, or myofibers, are packaged together into muscle fascicles, and numerous fascicles collectively form the skeletal muscle. Although the number and length of myofibers vary, common to each is the presence of multiple myonuclei. A sarcomere constitutes the basic contractile unit of a myofiber. One sarcomere is the distance between two adjacent Z-lines. The sarcomere is defined by thick (myosin) and thin (actin) filaments that are arranged in a repeating pattern along the length of the myofiber. These sarcomeres give rise to the striated appearance of the myofiber in electron micrographs. Skeletal muscle houses two distinct forms of mitochondria. The subsarcolemmal (SS) mitochondria lie beneath the sarcolemma of the myofiber and the inter-myofibrillar (IMF) mitochondria are found interspersed in between the myofibrils. It is well known that ~6 weeks of endurance training initiates changes in the performance characteristics and oxidative milieu of the myofiber. Endurance training mediates a “white-to-red” phenotype transition, which includes an increase in mitochondrial content and biogenesis, among many other adaptations. **B.** Exercise-induced mitochondrial biogenesis is the result of the coordinated interplay between the nuclear and mitochondrial genomes. Exercise triggers a multitude of signaling pathways, resulting in the downstream transactivation of regulatory proteins that control the transcription of nuclear genes encoding mitochondrial proteins (NUGEMPS), such as mitochondrial transcription factor A (Tfam). Subsequent to translation, nuclear gene products are targeted and translocated into the mitochondrion via the protein import machinery that spans the outer and inner mitochondrial membranes (OMM and IMM). Putative signals acting on mitochondrial transcription factors such as Tfam elicit transcription and translation of mitochondrial DNA (mtDNA)-encoded proteins. Nuclear- and mtDNA-transcribed proteins may act individually, or they can be integrated to form holoenzyme complexes, which are then incorporated into the electron transport chain (ETC), the site of oxygen consumption and energy production. See Color Plate 31.

Table 27-1. Changes in myonuclear domain with different models of skeletal muscle hypertrophy

Muscle	Hypertrophy model	Fiber type	Myonuclei number	Fiber cross-sectional area	Myonuclear domain size	Animal
Soleus	Synergist ablation (21 days)	N.A.	↑	↑	↔	Rat
Plantaris	Synergist ablation (10 weeks)	I, IIa	↑	↑	↔	Rat
		IIx/b	↑↑	↑	↓	
	Synergist ablation (3 months)	I	↑	↑	↔	Cat
		II	↑	↑	↔	
EDL	Synergist ablation	N.A.	↔	↑	↑	Rat
	Synergist ablation (4 weeks)	IIx, IIb	↑	↑	↔	
Vastus lateralis	Endurance exercise	N.A.	↑↑	↑	↓	Dog
Anterior latissimus dorsi	Weight (10% of body mass) attached to wing (30 days)	N.A.	↑	↑	↔	Quail
Trapezius	Several years of strength training	N.A.	↑	↑	↔	Human

Note: ↑, increase; ↓, decrease; ↔, no change.

activated T cells transcription factor family. The fusion of myoblasts with preexisting muscle fibers concomitantly increases myonuclear number, protein synthesis, and cytoplasmic volume, ultimately resulting in muscle fiber hypertrophy (Table 27-1). Despite the increase in myofiber size, the myonuclear domain, defined as the cytoplasmic myofiber volume per myonucleus, remains relatively constant. Table 27-1 summarizes current research on the changes in myonuclear domain size in response to imposed models of skeletal muscle hypertrophy.

Conversely, during muscle disuse and injury/disease, skeletal muscle may undergo atrophy, mediated by a decrease in protein synthesis and a concomitant increase in protein degradation through the activation of cell death pathways. Because skeletal muscle is multinucleated, the end result of apoptotic processes within this tissue is different when compared with that of mononucleated cells. The major difference is that apoptosis within skeletal muscle results in individual myonuclear loss, rather than degradation of the entire myofiber (Figure 27-2). However, the loss of individual myonuclei does not proceed without subsequently altering the phenotype of the apoptotic myofiber. When apoptosis strikes a myonucleus, the muscle fiber undergoes atrophy, which can result in an increase, maintenance, or reduction in the myonuclear domain size, regardless of fiber type, as shown in Table 27-2. According to the

myonuclear domain theory, each myonucleus controls a certain area of cytoplasm within a myofiber. Loss of myonuclei causes an associated decrease in overall cell volume so that the remaining myonuclei can continue to maintain the fiber. Along with myonuclear loss, there is a shift toward a fast-twitch fiber type as a result of an increase in the expression of fast and a decrease in the expression of slow myosin heavy chain (MHC) isoforms during atrophy. A variety of models that induce muscle atrophy, such as microgravity, spinal isolation, hind-limb unloading, and aging, have been shown to be associated with the loss of myonuclear number. However, recent work has challenged the theory of myonuclear loss with atrophy, rendering the debate controversial.

Two major conduits of myonuclear apoptosis have been elucidated during skeletal muscle atrophy: extrinsic pathway and the intrinsic pathway. The extrinsic pathway of apoptosis is regulated by the death receptor family of proteins. Ligand binding to the death receptor on the plasma membrane initiates a cascade of caspases, resulting in cell death. The intrinsic apoptotic pathway is mediated by the mitochondria.

2. MITOCHONDRALLY MEDIATED APOPTOSIS IN MUSCLE

Skeletal muscle has a high requirement for energy during contractile activity and consequently relies heavily on oxidative phosphorylation within the mitochondria

Table 27-2. Effect of skeletal muscle atrophy on myonuclear domain size

Muscle	Atrophy model	Fiber type	Myonuclei number	Fiber cross-sectional area	Myonuclear domain size	Animal
Diaphragm	Denervation	I, IIa	↔, ↔	↑, ↔	↑, ↔	Rat
		IIx, IIb	↔, ↔	↓, ↓	↓, ↓	
	Corticosteroid treatment	I, IIa	↔, ↔	↔, ↔	↔, ↔	
		IIx, IIb	↔, ↔	↓, ↓	↓, ↓	
Soleus	Aging	I	↓	↔	↑	Mouse
	Cold exposure (20 weeks)	I	↓	↓	↔	Rat
	Hindlimb suspension	I	↓	↓↓	↓	Rat
	Bed rest	I	↔	↓	↓	Human
	Spaceflight (14 days)	I	↓	↓↓	↓	Rat
		IIa	↔	↔	↔	
	Spinal cord isolation (2 months)	I	↓	↓↓	↓	Rat
		IIa	↔	↓	↓	
EDL	Aging	IIb	↓↓	↓	↑	Mouse
	Cold exposure (20 weeks)	N.A.	↔	↔	↔	Rat
TA	Spinal cord isolation	I, IIa,	↓	↓↓	↓	Rat
Gastrocnemius	(4 days and 60 days)	IIx, IIb	↓	↓↓	↓	Rat

Note: ↑, increase; ↓, decrease; ↔, no change.

to meet the functional demands placed on it. Mitochondria function to donate electrons to molecular oxygen to create energy in the form of adenosine triphosphate (ATP) so that muscular contraction can occur. Along with providing the majority of energy needed to fulfill many essential cellular processes, these vital organelles are also intimately involved in the regulation of apoptosis. As outlined in detail elsewhere in this volume, mitochondria are prime transducers of apoptosis for several reasons. First, mitochondria contain a number of proapoptotic proteins such as apoptosis inducing factor (AIF), endonuclease G (Endo G), and cytochrome c, which can be released on receipt of an appropriate signal. Furthermore, mitochondria are the main producers of reactive oxygen species (ROS). ROS are formed when an electron from complex I or III of the ETC is inappropriately donated to oxygen. This results in the formation of a superoxide anion (O_2^-), which is swiftly converted into hydrogen peroxide (H_2O_2). During resting conditions, 1% to 2% of the total oxygen consumed is

converted into ROS, and this percentage can increase during muscle disuse, injury, or disease. If left unquenched, ROS can induce damage and initiate apoptosis.

2.1. Skeletal muscle apoptotic susceptibility

Apoptosis in skeletal muscle is unique compared with the majority of other tissues for several reasons. As alluded to earlier, apoptosis in skeletal muscle leads to the degradation of individual myonuclei rather than the entire fiber. This may produce changes in the myonuclear domain, ultimately resulting in muscle atrophy (Table 27-2). Second, muscle tissue is composed of a variety of specialized fiber types that accommodate distinctly different levels of mitochondrial content and functional activity. Slow-twitch (type I) muscle fibers contain more mitochondria and more myonuclei as compared with fast-twitch (type II) fibers. Thus the extent of the apoptotic susceptibility of individual

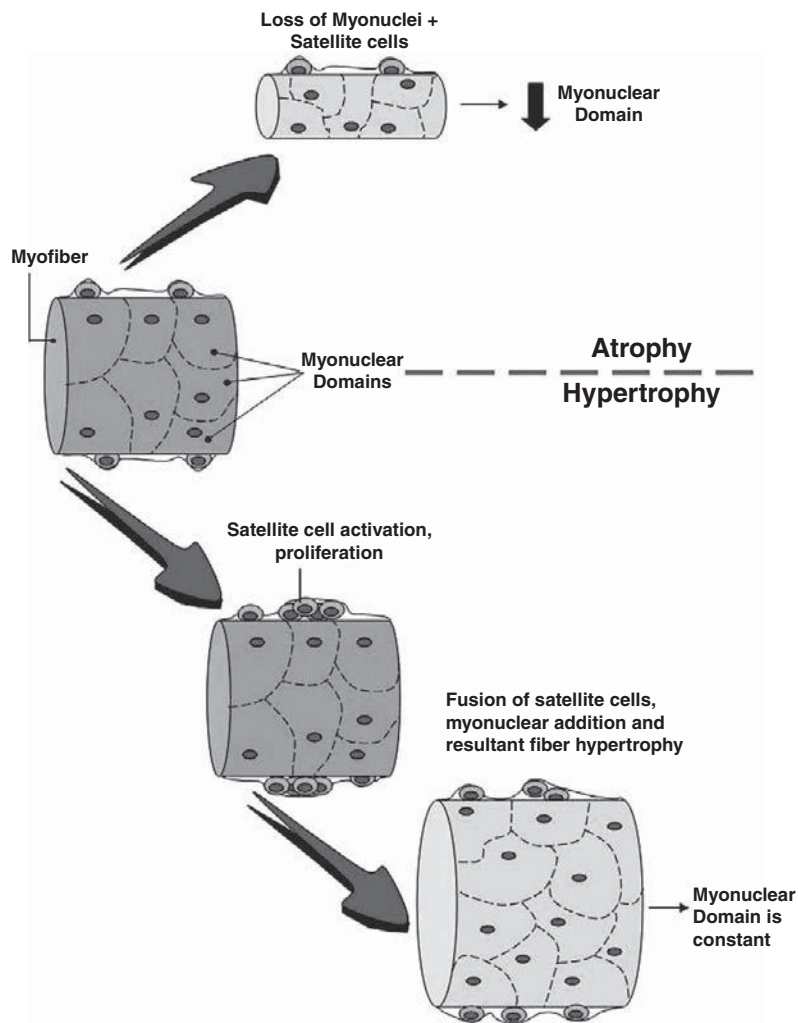


Figure 27-2. Myonuclear domains during muscle hypertrophy and atrophy. Myofibers are large, multinucleated cells surrounded by small, mononucleated satellite cells. As a result of a stimulus leading to muscle hypertrophy, satellite cells proliferate, fuse, and donate myonuclei to existing myofibers. This results in an increase in myonuclear number and cytoplasmic volume, producing a larger myofiber, while leaving the myonuclear domain size relatively constant (see Table 27-1). Conversely, conditions leading to muscle atrophy (e.g., muscle denervation/disuse) are accompanied by a reduction in satellite cell number. Whether a decline in myonuclear number occurs is controversial (see Table 27-2, Bruusgaard et al.), and it likely does not occur to the same extent as the reduction in cytoplasmic volume. This manifests as a decrease in myonuclear domain size and consequently leads to a smaller myofiber cross-sectional area (see Table 27-2). See Color Plate 32.

muscle fibers varies and is specific to the type of cell death signal evoked, the duration of the signal, and the organism under investigation. Third, muscle fibers contain two distinct mitochondrial subfractions, SS and IMF mitochondria. These two mitochondrial subpopulations appear to regulate apoptosis differently. SS mitochondria produce more ROS and have a higher Bax/Bcl-2 ratio compared with IMF mitochondria. In contrast, IMF mitochondria have a greater rate of cytochrome c and AIF release. However, because the SS and IMF mitochondrial subfractions make up approximately 20% and 80% of the total mitochondrial content within a muscle cell,

respectively, it is likely that the IMF subfraction is the more important contributor to skeletal muscle apoptotic signaling.

3. EVIDENCE OF APOPTOSIS DURING MUSCLE DISUSE

Investigations of how prolonged disuse affects skeletal muscle began in the 1950s and are currently still a research area that garners significant attention. Results from these studies have shown that chronic muscle disuse results in a reduction in skeletal muscle mass. More recent studies have documented decreases in skeletal muscle oxidative capacity and an increase in cellular susceptibility to apoptosis. Furthermore, chronic muscle disuse has been shown to have an immediate impact on SS mitochondrial content, whereas longer periods of disuse are required to affect IMF mitochondrial content. Currently, there are a number of different models that represent reduced contractile activity, such as space flight, limb immobilization, denervation, and bed rest. Although many of the signaling pathways associated with apoptosis have been well characterized, there still remain many questions regarding the exact contribution of apoptosis to disuse-induced muscle atrophy.

3.1. Mitochondrially mediated apoptosis during chronic muscle disuse

Chronic muscle disuse leads to a number of adaptations within skeletal muscle that increase the susceptibility of mitochondria to apoptosis. In particular, prolonged muscle disuse leads to a reduction in the expression of cytochrome c mRNA in both slow- and fast-twitch muscles. This reduction exceeds the rate of overall muscle protein loss, suggesting that inactivity specifically targets mitochondrial proteins. In conjunction with a decrease in mitochondrial protein expression, a number of oxidative enzymes such as succinate dehydrogenase, citrate synthase, cytochrome c oxidase, and malate dehydrogenase are all decreased with reductions in contractile

activity. Further, mitochondria from disused skeletal muscle possess a reduced capacity to generate ATP per gram of muscle and display an overall decrease in mitochondrial function. This has a pronounced effect on skeletal muscle performance, leading to increased fatigability and a poor endurance capacity.

Disuse-induced decrements in mitochondrial oxidative capacity also contribute to the activation of mitochondrially mediated apoptosis via the enhanced production of ROS. ROS have been shown to increase in slow-twitch and in fast-twitch muscles during 28 days of hind-limb unloading. In addition, the ROS metabolizing enzymes manganese superoxide dismutase (MnSOD), catalase, and glutathione peroxidase are all reduced in their expression, and this increases the impact of the augmented ROS production. Similar results have also been demonstrated using chronic skeletal muscle denervation, demonstrating that this adaptation occurs in various models of muscle disuse. Mitochondria from denervated skeletal muscle exhibit an increased rate of mitochondrial permeability transition pore (mtPTP) opening kinetics, indicating an increase in apoptotic susceptibility. Additionally, chronic muscle disuse also alters the expression of pro- and antiapoptotic mitochondrial proteins. During denervation-induced muscle disuse, there is an increase in the Bax:Bcl-2 ratio, which favors the formation of the mtPTP. Thus chronic muscular inactivity leads to a number of proapoptotic adaptations, which include (1) a reduction in overall mitochondrial function, (2) an increase in ROS production, and (3) a faster rate of mtPTP formation. Interestingly, the elevated expression of Bax has further significance as this protein is able to homodimerize on the outer membrane of the mitochondria, forming the mitochondrial apoptosis channel (MAC). MAC is capable of facilitating the release of small proteins such as cytochrome c, but not larger proteins like AIF. Thus the implications of an increase in Bax protein expression are twofold: (1) Bax assists in the formation of the mtPTP, and (2) Bax can form its own pore, facilitating further proapoptotic protein release.

The disuse-induced increase in ROS production likely promotes the release of proapoptotic proteins and the fragmentation of myonuclear DNA. Using denervation as a model of disuse, a 40% increase in cytosolic cytochrome c and a 10-fold increase in AIF localized within the cytosol was observed. This increase in cytochrome c was paralleled by a 500% increase in cleaved caspase-3, indicating the activation of caspase-dependent apoptosis, and a 100% increase in DNA fragmentation. Other forms of muscle disuse such as hind-limb suspension potentiate similar increases in

the caspase-independent apoptotic pathway. Within 12 hours of disuse induction, significant increases in the localization of Endo G within the nucleus and concomitant increases in DNA fragmentation were observed. Collectively, these results suggest that chronic muscle disuse induces proapoptotic changes within muscle, which lead to an increase in mitochondrial apoptotic susceptibility and myonuclear apoptosis and contribute to skeletal muscle atrophy.

4. APOPTOSIS IN MUSCLE DURING AGING AND DISEASE

In addition to muscle disuse, apoptosis in skeletal muscle is also enhanced in various pathological conditions such as aging, type 2 diabetes mellitus, chronic heart failure, and cancer. Even though skeletal muscle is not necessarily the tissue responsible for the initial clinical manifestations of these diseases, most, if not all, of these pathological conditions are associated with mitochondrial dysfunction within skeletal muscle. This elevation in apoptosis contributes to a reduction in muscle mass, and the concomitant manifestation of mitochondrial dysfunction compromises endurance capacity, thereby severely affecting the quality of life of the affected individual.

4.1. Aging

Muscle atrophy that occurs with increasing age is commonly referred to as sarcopenia. The decline in muscle mass with age has been attributed to a number of external factors such as motor unit rearrangement, hormonal status, and reduced satellite cell recruitment. Although much research has been conducted in this area, the signaling pathways that regulate this type of cell death remain elusive. Studies have documented muscle atrophy in a multitude of muscles with differing fiber type content such as the slow-twitch soleus, as well as the predominantly fast-twitch plantaris, gastrocnemius, and extensor digitorum longus muscles. Although the reduction in muscle mass is observed in each fiber type, the process is differentially regulated. Fast-twitch (type II) muscle fibers appear to be more susceptible to programmed cell death during the normal physiologic condition of aging. This likely occurs because type II fibers have less myonuclei per fiber compared with slow-twitch (type I) fibers, and/or the regular distribution of myonuclei is impaired, thus increasing transport distances of de novo gene products. Additionally, a selective loss of motor unit innervation to type II fibers occurs with advancing age. Thus an aged muscle will ultimately retain a higher proportion of type I MHC-containing

fibers compared with that same muscle before atrophy, and this is due to a preferential loss of fast-twitch type II fibers.

Recently, an increased incidence of apoptosis and enhanced expression of proapoptotic proteins has been documented in aged skeletal muscle. Skeletal muscle is more susceptible to age-induced deterioration because it is postmitotic. Although satellite cells are capable of replacing myonuclei that have been lost because of apoptosis, both the percentage of satellite cells and their proliferative capacities decrease with advancing age. Furthermore, mitochondrial dysfunction likely plays an important role in increasing the susceptibility of muscle to apoptosis, because mitochondria from senescent animals have enhanced ROS production, elevated levels of mtDNA mutations, and an augmented release of cytochrome c and Endo G as compared with their young counterparts. Transgenic mice expressing a proofreading-deficient version of mitochondrial DNA polymerase γ accumulate a high degree of mtDNA mutations and exhibit several age-related phenotypes, such as sarcopenia. Interestingly, apoptosis was observed to be one of the potential mechanisms responsible for the mtDNA mutation-induced sarcopenia in these animals. This suggests that mtDNA mutations, which lead to mitochondrial dysfunction and promote apoptosis, may play a pivotal role in age-related phenotypes such as skeletal muscle mass loss.

4.2. Type 2 diabetes mellitus

Insulin resistance and type 2 diabetes mellitus (T2DM) are common complications of obesity and a sedentary lifestyle. These conditions are characterized by elevated circulating concentrations of fatty acids (FAs) and aberrant FA metabolism, resulting in increased FA storage in nonadipose tissues such as skeletal muscle. These FAs are stored in the form of triglycerides, diacylglycerol, and ceramides. Recent evidence supports a role for FA-induced skeletal muscle apoptosis, termed lipoapoptosis, which results from FA overload. Exposure of muscle cells to the saturated FA palmitate results in apoptosis, implicating high levels of FAs in pathological conditions such as obesity and T2DM as a contributor to skeletal muscle programmed cell death. FAs can increase the production of ROS, further augmenting the susceptibility of skeletal muscle toward apoptosis. A growing body of evidence implicates oxidative stress, resulting from ROS-induced mitochondrial dysfunction, as a major contributor to skeletal muscle apoptosis. ROS-induced mtDNA damage has been

demonstrated to impair glucose utilization and insulin resistance in skeletal muscle. mtDNA mutations are elevated within skeletal muscle of T2DM patients, presumably in concert with an increase in ROS production as a result of high levels of FA. A reduction of mtDNA to levels of < 10% of normal cells using ethidium bromide has been illustrated to result in altered insulin signaling, as evidenced by a reduced expression of insulin receptor substrate-1 (IRS-1), decreased insulin-stimulated phosphorylation of IRS-1 and protein kinase B, and attenuated glucose transporter-4 translocation to the plasma membrane. This implicates a vicious cycle, whereby obesity results in FA accumulation within skeletal muscle, leading to increased ROS production. This elevation in oxidative stress can damage mtDNA and reduce the oxidative capacity of skeletal muscle by both decreasing mitochondrial function and reducing muscle mass via apoptosis. The reduction of skeletal muscle mass and oxidative capacity further exacerbates FA accumulation, thereby continuing the cycle. Additionally, because skeletal muscle is the main tissue responsible for glucose disposal from the circulation, reductions in the amount of skeletal muscle as a result of enhanced apoptosis will further propagate the clinical symptoms of T2DM.

4.3. Cancer cachexia

Severe muscle wasting, termed cachexia, occurs in the majority of cancer patients before death and accounts for nearly one-third of cancer deaths. Individuals suffering from cancer cachexia can lose up to 80% of their skeletal muscle mass. Fortunately, muscle wasting does not occur in all forms of cancer. For example, breast cancer and non-Hodgkin's lymphoma patients rarely develop cachexia. However, gastrointestinal cancer patients are highly prone to muscle wasting. Although the loss of muscle mass observed during cancer cachexia can be jointly attributed to a reduction in protein synthesis and elevated protein degradation, the majority of muscle wasting during the advanced stages of cancer is due to increased protein degradation. The ubiquitin-proteasome pathway and apoptosis have been demonstrated to be contributing factors to skeletal muscle degeneration in cancer cachexia.

Skeletal muscle apoptosis is a secondary clinical manifestation of cancer patients. Increased DNA fragmentation, a hallmark of apoptosis, has been observed in the gastrocnemius muscle of mice and rats inoculated with tumorigenic liver and lung cells and in the rectus abdominis muscle of human gastrointestinal cancer patients. Higher levels of cytochrome c release from

mitochondria, along with enhanced caspase activation in muscle from cachectic mice, also supports a role for apoptosis during cancer cachexia. Evidence suggests that the cachectic response is mediated by inflammatory cytokines such as tumor necrosis factor- α (TNF- α). TNF- α is implicated as a major contributor toward skeletal muscle apoptosis in cancer patients. This cytokine can induce apoptosis by regulating the dephosphorylation and subsequent degradation of the antiapoptotic protein Bcl-2. Additionally, TNF- α mediates caspase-dependent programmed cell death via binding to its receptor on the sarcolemmal membrane of myofibrils. In conjunction with elevated levels of apoptosis, TNF- α potentiates an increase in proteolysis, as evidenced by increased expression of ubiquitin and proteasome subunits. Thus available data strongly indicate a role for apoptosis in cancer cachexia.

4.4. Chronic heart failure

Apoptosis occurs not only in the myocardium, but also in the skeletal muscle of chronic heart failure (CHF) patients. Just like individuals suffering from cancer cachexia, CHF patients also have elevated levels of inflammatory cytokines such as TNF- α . TNF- α levels have been documented to be the strongest predictors of the degree of muscle loss occurring in CHF patients. Muscle biopsies from human CHF patients exhibit elevated markers of apoptosis such as DNA fragmentation and caspase-3 cleavage. CHF patients also have elevated levels of growth hormone (GH) with concomitant decreases in insulin-like growth factor-I (IGF-1), suggesting the presence of GH resistance. IGF-1 inhibits apoptosis in cardiac cells by reducing the expression of Bax and caspase-8, as well as the activity of caspase-3. Thus the reduction of circulating IGF-1 levels in CHF patients results in an attenuation of apoptotic repression, thereby enhancing apoptotic susceptibility within skeletal muscle. Additionally, CHF patients exhibit high amounts of oxidative stress, which may further contribute to the induction of apoptosis.

A key symptom of CHF patients is exercise intolerance, which is largely due to ventricular dysfunction. Ultrastructural and functional analyses have also revealed abnormalities in skeletal muscle mitochondria from CHF patients, suggesting that mitochondrial dysfunction within skeletal muscle is a contributing determinant to the clinical symptoms, and the reduced quality of life, associated with CHF.

Given the plasticity of skeletal muscle, it is not surprising that apoptotic susceptibility and mitochon-

drial deficits observed during muscle disuse and disease can be altered by exercise. Although exercise-induced adaptations are dependent on the type, intensity, duration, and frequency of exercise training, emerging evidence indicates that exercise/chronic contractile activity can reduce apoptotic susceptibility within skeletal muscle. This exercise-mediated repression of apoptosis may prove to be an important therapeutic intervention for many diseases that are associated with apoptotic induction within this tissue.

5. EFFECT OF ENDURANCE EXERCISE/CHRONIC CONTRACTILE ACTIVITY ON APOPTOSIS

Skeletal muscle is composed of a variety of specialized fiber types containing a continuum of mitochondrial contents, a variable that adapts readily to changing conditions of contractile activity. Indeed, the major biochemical adaptation that occurs within the muscle with exercise training is an increase in mitochondrial content, leading to functional improvements in fatigue resistance. Because mitochondria are closely associated with apoptosis, it is logical to presume that exercise may modulate rates of apoptosis. The effects of chronic contractile activity on apoptosis have been investigated using different models of endurance exercise training such as voluntary wheel running, treadmill training, and chronic low frequency electrical stimulation using both acute and chronic training models.

Although acute exercise has been demonstrated to enhance apoptosis in muscle, a plethora of data indicates that chronic exercise may have a protective effect on muscle apoptotic susceptibility. Treadmill running over a period of 8 weeks enhanced the antiapoptotic (Bcl-2, XIAP) and antioxidant (HSP70 and MnSOD) protein levels, with a simultaneous decrease in apoptotic proteins (Apaf-1 and Bax) and DNA fragmentation in the skeletal and cardiac muscle of trained compared with control animals. In agreement with these data, it was recently illustrated that chronic contractile activity, imposed via electrical stimulation, resulted in mitochondrial biogenesis and a concomitant reduction in mitochondrial apoptotic susceptibility. This was indicated by a decrease in ROS production within the IMF mitochondrial subfraction, along with reduced levels of cytochrome c and AIF release from SS and IMF mitochondria isolated from chronically stimulated muscle. As opposed to endurance exercise, resistance training does not alleviate apoptosis, despite attenuating the loss in muscle mass, during hind-limb unloading as a model of muscle atrophy.

The exercise-mediated attenuation of apoptotic susceptibility is also observed during muscle disuse conditions such as aging. Treadmill training in old rats improved exercise capacity and muscle strength and reduced the age-related increase in apoptotic markers such as an increase in the Bax:Bcl-2 ratio, caspase activation, and DNA fragmentation. However, endurance training in mitochondrial myopathy patients reduced the expression of DNA repair proteins and produced higher oxidative damage despite an increase in anti-apoptotic MnSOD protein expression, indicating that more research is required to establish whether exercise training can be a beneficial therapeutic modulation for patients with mtDNA defects and other muscle myopathies.

6. CONCLUSION

Skeletal muscle is a versatile tissue that adapts phenotypically to both decreases and increases in contractile activity. During muscle disuse or disease, reductions in muscle mass, strength, mitochondrial content, and endurance performance are observed. In contrast, endurance exercise training elevates muscle mitochondrial content, and this appears to attenuate mitochondrially mediated apoptosis. Apoptosis plays a well-established role in muscle disuse conditions such as aging and in pathological conditions such as T2DM and cancer cachexia. Further research is warranted to fully elucidate the molecular regulation of apoptosis in skeletal muscle and to fortify a role for exercise training as an ideal therapeutic intervention to preserve muscle function during chronic muscle disuse and disease.

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1. INTRODUCTION

During life we are persistently exposed to environmental hazards. A first protective barrier is provided by the skin, protecting us against water loss and external physical, chemical, and biological insults such as wounding, UVB radiation, and microorganisms. The skin consists of an outer squamous epithelium, the epidermis, and an inner connective tissue, the dermis. The barrier is mainly constituted by the epidermis, which is continuously rejuvenated as a result of mitotic activity of the stem cells in the basal layer that provide new keratinocytes. Upon withdrawal from the cell cycle, basal keratinocytes detach from the basement membrane and undergo a terminal differentiation program to become corneocytes in the outer layers of the epidermis (Figure 28-1). At the transition from the granular to cornified layer, an increase in intracellular Ca^{2+} activates transglutaminases, which cross-link different structural proteins beneath the plasma membrane to form the cornified envelope. At the final stage of differentiation, the keratinocytes lose their organelles, including the nucleus, and become the dead, flattened corneocytes. This cell death program, called cornification, has to be well orchestrated because the dead cells act as an essential barrier and fulfill a specific function. Finally, corneocytes are shed from the skin by a process called desquamation. Melanocytes, also residing in the epidermis, are neurectoderm-derived cells that produce melanin, which provides skin pigmentation. Imbalances in the delicate physiologic turnover of proliferating or differentiating keratinocytes can result in the disturbance of the skin barrier function and are reflected in many skin disorders. In addition, improper removal of damaged cells by the terminal differentiation program can result in cancerous lesions.

2. CELL DEATH IN SKIN HOMEOSTASIS

2.1. Cornification and apoptosis

To form cornified envelopes at the outermost epidermal layer, keratinocytes must undergo cell death. Although this process shows parallels with apoptosis, it should be distinguished from it based on morphological and biochemical differences. Apoptosis is a rapid process occurring in single cells that are immediately engulfed by other cells such as macrophages. In vivo, terminal keratinocyte differentiation is a process simultaneously occurring in a whole cell layer, taking up to 2 weeks, and the dead cells fulfill an essential function in the skin barrier before they are shed. It was proposed that apoptosis is blocked during differentiation to prevent premature apoptotic cell death during keratinocyte differentiation and to allow correct cornification. In keratinocytes, the $\beta 1$ -integrins mediate adhesion to the extracellular matrix and regulate the initiation of terminal differentiation and cell-detachment apoptosis, or anoikis. Epidermal keratinocytes differentiate when they detach from the basement membrane and migrate to the suprabasal layers. This differentiation signal is transduced by unoccupied $\beta 1$ -integrins. However, in experimental keratinocyte suspension cultures, unoccupied $\beta 1$ -integrins induce an apoptotic signaling cascade that results in upregulated Bax expression and mitochondrial damage, eventually leading to cell death. This observation implies the existence of an innate survival mechanism required to maintain the viability of suprabasal cells in vivo (Figure 28-2). Activation of the nuclear factor kappa B (NF- κ B) transcription factor is one of the antiapoptotic mechanisms that is initiated during keratinocyte differentiation. NF- κ B is a complex formed by homo- and heterodimerization

of the NF- κ B family members p50, p52, RelA (p65), RelB, and c-Rel. The I κ B kinase (IKK) complex, consisting of the IKK α and IKK β catalytic subunits and the NEMO (also known as IKK γ) regulatory subunit, phosphorylates the NF- κ B inhibitory I κ B proteins, targeting them for degradation. This leads to the release of the NF- κ B transcription factor and unmasking of the nuclear translocation signal sequence, nuclear translocation, and transcriptional activity of NF- κ B. Active NF- κ B is most often composed of p50/RelA heterodimers. NF- κ B is not activated in proliferating epidermal cells, but is activated and translocated to the nucleus on differentiation, as witnessed by nuclear translocation of the RelA subunit. The relevance of this observation is challenged by the fact that mice with epidermis-specific deletion of RelA are phenotypically identical to wild-type mice, suggesting redundancy in the use of NF- κ B subunits. However, combined deletion of RelA and c-Rel did not affect epidermal keratinocyte differentiation, but basal cells showed reduced proliferation capacity. In the epidermis, the expression of NF- κ B-dependent antiapoptotic proteins such as c-IAP-1, c-IAP-2, TRAF1, and TRAF2 is increased in differentiating keratinocytes. In addition, transgenic over-expression of dominant-negative I κ B α in murine skin results in premature spontaneous cell death exhibiting apoptotic properties. The latter data may point to an important role for NF- κ B in the protection of keratinocytes against apoptosis during terminal differentiation.

However, more recent evidence suggests that the protection provided by NF- κ B may only be true under inflammatory conditions characterized by increased cytotoxic cytokine levels, such as tumor necrosis factor (TNF). Genetic deletion of crucial components for the NF- κ B signaling pathway can result in premature keratinocyte apoptosis. This is observed in NEMO-deficient mice, a model for incontinentia pigmenti (IP). In humans, this male lethal disorder is caused by mutations in the X-linked NEMO gene. Females develop blisters and an inflammatory response in the

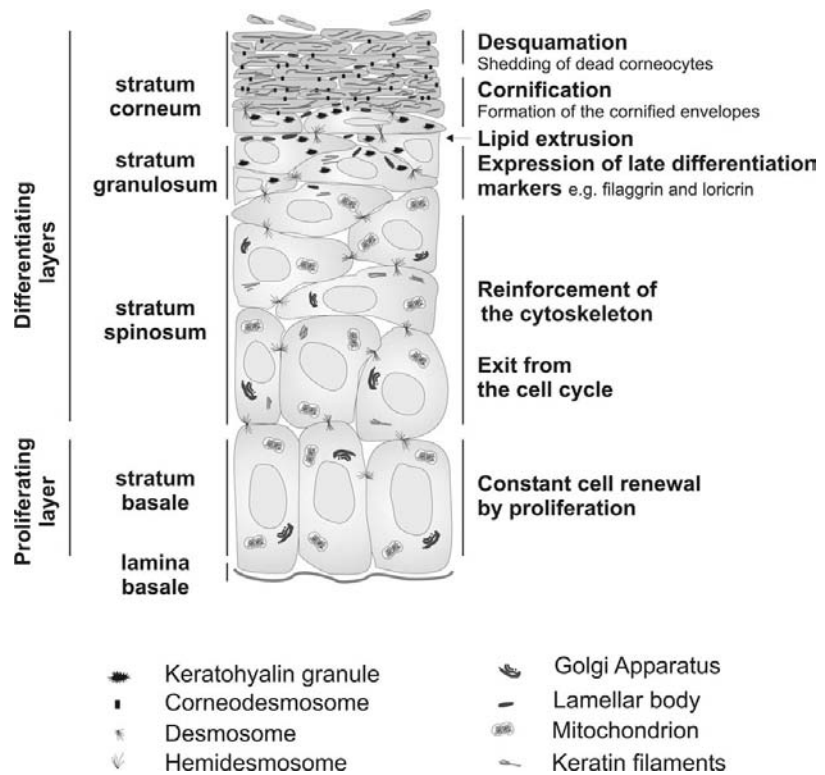


Figure 28-1. The epidermis. Depending on the differentiation stage of the keratinocytes, the epidermis is subdivided into different sublayers, or *strata*. The basal layer (stratum basale) is a monolayer of cuboidal cells that are attached to the basement membrane (lamina basalis) through hemidesmosomes. This layer contains proliferating stem cells that endlessly provide the stratified epithelium with new cells. In the intermediate spinal layer (stratum spinosum), the cells reinforce their cytoskeletal keratin filament network, and adjacent cells interact via many desmosomes, a specialized type of cell junction, to resist physical trauma. In the granular layer (stratum granulosum), the keratinocytes become more flattened and express certain proteins such as profilaggrin and loricrin, which aggregate to form the typical keratohyalin granules. In addition, lipids are produced and stored in lamellar bodies. At the transition between the granular and cornified layer (stratum corneum), the cells lose their nucleus and other organelles; proteins are cross-linked at the inner side of the cytoplasmic membrane to form a cornified envelope (CE) and are connected by corneodesmosomes (modified desmosomes containing corneocyte-specific components such as corneodesmosin). Lipids are extruded to form a water-repelling envelope around the CE, thereby assuring an adequate permeability barrier function of the mammalian epidermis. Finally, the dead corneocytes or cornified envelopes are discarded into the environment during desquamation.

epidermis. This is followed by hyperkeratotic lesions that usually spontaneously resolve, leaving behind areas of hyperpigmentation. Premature apoptosis is also seen in skin of transforming growth factor β -activated kinase 1 (TAK1) deficient mice. TAK1 functions downstream of inflammatory receptors to activate c-Jun N-terminal kinase (JNK) as well as NF- κ B. For NEMO- and TAK1-deficient mouse models, it was demonstrated that the keratinocytes are more sensitive to TNF-induced apoptosis, but why TNF levels are augmented is not clear. In addition, increased TNF levels are also observed in skin-specific IKK β -deficient mice.

Interestingly, both IKK β - and TAK1-deficient mice develop severe skin inflammation shortly after birth,

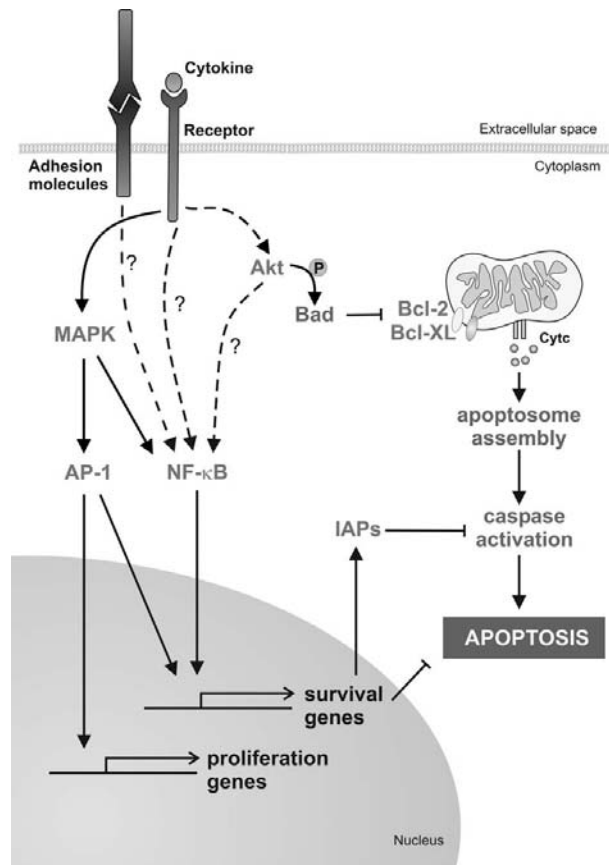


Figure 28-2. Antiapoptotic mechanisms during skin homeostasis. During epidermal differentiation, several prosurvival pathways are activated, including the MAPK, NF- κ B, and Akt pathway. The MAPK and Akt pathway can be activated by growth factor receptors, such as epidermal growth factor receptor. How NF- κ B is triggered under homeostatic conditions in the skin is currently not clear. It has been suggested that the changed suprabasal keratinocyte interactions mediated by means of adhesion molecules could be involved in NF- κ B activation in these layers. MAPK signaling can result in activator protein-1 (AP-1) transcription factor activation, which controls proliferation. There exists interplay between Akt and NF- κ B because it has been documented that Akt can also activate the NF- κ B pathway and NF- κ B can induce Akt. However, the molecular details of this interplay are not well known. NF- κ B induces the transcription of antiapoptotic genes, whereas Akt can inhibit the proapoptotic protein Bad by phosphorylating it. Phosphorylated Bad can no longer bind and inhibit the prosurvival Bcl-2 family members Bcl-2 and Bcl-XL. Apoptotic signaling can lead to mitochondrial outer membrane permeabilization and cytochrome c release. Cytochrome c will bind (together with (d)ATP) to Apaf-1, resulting in apoptosome assembly and caspase-9 activation. At least in hair follicle cells and melanocytes, it has been shown that Bcl-2 protects these cells against premature apoptosis in homeostatic conditions. Inhibitor of apoptosis proteins (IAPs) can inhibit caspases, thereby hampering the apoptotic capacity of the cell. In addition, IAPs are also transcriptional targets of NF- κ B.

which is TNF-R1 and/or JNK dependent. The initiation of skin disease during the first few days after birth may indicate the involvement of environmental factors. These observations imply that NF- κ B is of major impor-

tance for the maintenance of a well-balanced immune system in the skin. Although the exact mechanism of inflammation initiation remains unclear, ablation of NF- κ B activators from epidermal keratinocytes seems to disturb the immune balance of the skin, resulting in an inflammatory response that is marked by the induction of inflammatory cytokines. Alternatively, mechanisms related to developmental changes of the skin at this early age might be involved in disease initiation. In contrast to IKK β , IKK α seems to play a crucial role in skin barrier formation, but independent from its NF- κ B signaling function. Besides epidermal differentiation, development of hair follicles and limbs and tail also requires IKK α .

Analogous to the skin phenotype observed by ablation of essential NF- κ B activating genes, increased NF- κ B activation by deletion of I κ B α leads to severe skin inflammation, which is TNF, lymphotoxin, and RelA dependent. Tissue-specific deletion of I κ B α emphasizes the cross-talk between epidermal and immune cells in the development of skin inflammation. In addition, ablation of RelA from epidermal keratinocytes completely rescued the inflammatory skin phenotype of I κ B α -deficient mice. It is noteworthy that in case of over-activation of NF- κ B, the inflammatory skin phenotype is T-cell dependent, whereas inactivation of NF- κ B results in T-cell independent inflammation. These findings emphasize the important role of NF- κ B activation in both keratinocytes and lymphocytes in the development of skin inflammation and underscore the important role of the epidermis as a sensor of inflammatory stimuli.

Other pathways that prevent cell death in the skin are the PI3K/AKT and mitogen-activated protein kinase (MAPK) pathway, both activated by epidermal growth factor receptor. Genetic deletion of Akt1 or Akt1/Akt2 in the mouse results in thinner skin with fewer hair follicles. This phenotype is explained by reduced keratinocyte proliferation. In contrast, knockdown of Akt1, but not of Akt2, in in vitro reconstituted human epidermis results in premature keratinocyte apoptosis and thinner suprabasal layers. Whether the observed difference in Akt implication between the mouse and human epidermis reflects species differences or the fact that in the developing mouse epidermis compensatory effects result in a milder phenotype is currently not clear. Akt can block apoptosis through phosphorylation of the proapoptotic Bcl-2 family member BAD, leading to its inactivation. Importantly, there exists an interplay between the PI3K/Akt and NF- κ B pathway, because Akt is believed to activate NF- κ B through IKK α and β and NF- κ B can induce Akt. The possible link

between Akt and NF- κ B was not explored in the Akt1 and Akt2 double-deficient Akt mice.

In both apoptosis and keratinocyte differentiation, protease activity is indispensable. Several skin-specific proteases are required for cornification. The apoptotic caspases, such as caspase-3, -6, -7, and -9, are probably not involved in cornification because they are not activated during differentiation, and deficiency of their genes in the mouse does not result in apparent skin abnormalities. The involvement of caspase-3 in embryonic skin homeostasis remains the subject of debate, because both the absence and the occurrence of caspase-3 activation has been reported in the developing epidermis in mice. Caspase-14, however, is clearly required for normal skin development. This caspase family member is nonapoptotic, almost exclusively expressed in the suprabasal epidermal cells, and becomes activated during cornification. Caspase-14-deficient mice have defects in proper stratum corneum formation resulting in increased transepidermal water loss and increased sensitivity to UVB irradiation as compared with wild-type mice. Profilaggrin, a direct caspase-14 substrate, is not correctly degraded in caspase-14-deficient mice, whereas this is a crucial event in the formation of a completely functional skin barrier. Filaggrin is not only a major CE structural protein, it is also important in skin hydration. In the upper cornified layers of wild-type mice, in contrast to caspase-14^{-/-} mice, filaggrin is further degraded, and this process gives rise to natural moisturizing factors (NMFs) and urocanic acid, a UVB scavenger.

The distinction between apoptotic cell death and cornification is also made at the mitochondrial level. The release of cytochrome c during in vitro keratinocyte terminal differentiation has been reported, but this did not result in apoptosis, although the apoptosome components Apaf-1 and caspase-9 are present in epidermal keratinocytes. Instead, it was suggested that cytochrome c release during keratinocyte differentiation leads to transcription factor activation and gene expression. Anti-apoptotic Bcl-2 is downregulated and proapoptotic Bax and Bak are induced in the suprabasal layers of the epidermis, but no abnormalities in skin differentiation have been reported for any of the Bcl-2 family member knockouts. One exception is *bcl-2*, which is implicated in hair follicle turn-over. Knockout or transgenic mice for this gene show a retardation or acceleration in the first hair follicle growth phase, respectively. In addition, graying occurs during the hair follicle catagen or apoptosis-driven involution phase in Bcl-2^{-/-} mice, probably due to melanocyte apoptosis.

2.2. Death receptors in the skin

The members of the TNF receptor (TNF-R) superfamily are characterized by extracellular cysteine-rich domains that bind their respective ligands and intracellular interaction motifs, such as the death domain (DD) and the TNF-receptor associated factor (TRAF)-binding domain. In general, these receptors are capable of initiating signaling cascades that lead to transcription factor activation and/or cell death. The TNF-R superfamily comprises the so-called death receptors (DRs), namely TNF-R1, Fas, TNF-related apoptosis-inducing ligand (TRAIL) R1 and R2, TRAMP, DR6, EDAR, and p75^{NTR}, which have a cytoplasmic death domain (DD). TNF-R1, Fas, and TRAIL-R and their respective ligands TNF, FasL, and TRAIL are expressed by keratinocytes and have been shown to be able to induce cell death in keratinocytes. In normal conditions, the ligands are poorly expressed or may remain intracellular so that no spontaneous apoptosis occurs, but in pathological conditions (see [Section 3](#), Cell Death in Skin Pathology), activation of these receptors leads to keratinocyte apoptosis.

The nonapoptotic TNF-R family members EDAR, XEDAR, and TROY trigger the ectodysplasin (EDA) pathway, which is of major importance for skin appendage development. The ligands for EDAR and XEDAR (EDA-A1 and EDA-A2) are splice variant products of the same gene *ED1*, whereas TROY is still an orphan receptor. The exact function of the EDA pathway in appendage formation is not known, but mutations in components of the EDA pathway result in hypohidrotic ectodermal dysplasia (HED), manifested by absence or defective formation of hair, teeth, fingers or toes, and sweat glands, which causes overheating. The same phenotype is found in mice that are mutant for EDA or EDAR or its downstream signaling molecules EDAR-associated death domain protein (EDARADD) and TRAF6. Surprisingly, the EDAR death domain receptor does not signal to cell death, but rather to NF- κ B to prevent premature cell death during the hair follicle catagen involution phase. X-linked HED (XLHED) can be caused by missense mutations in *ED1*, resulting in dysfunctional EDA ligand. In mice it was shown that the HED phenotype can be rescued by expression of the correct ligand or prenatal recombinant protein administration of Fc:EDA-A1, a fusion protein of an IgG1 Fc domain with EDA-A1, which facilitates delivery through the placenta and increases the molecule stability. Postnatal injection of Fc:EDA1 in mice and canine XLHED models gave very promising results.

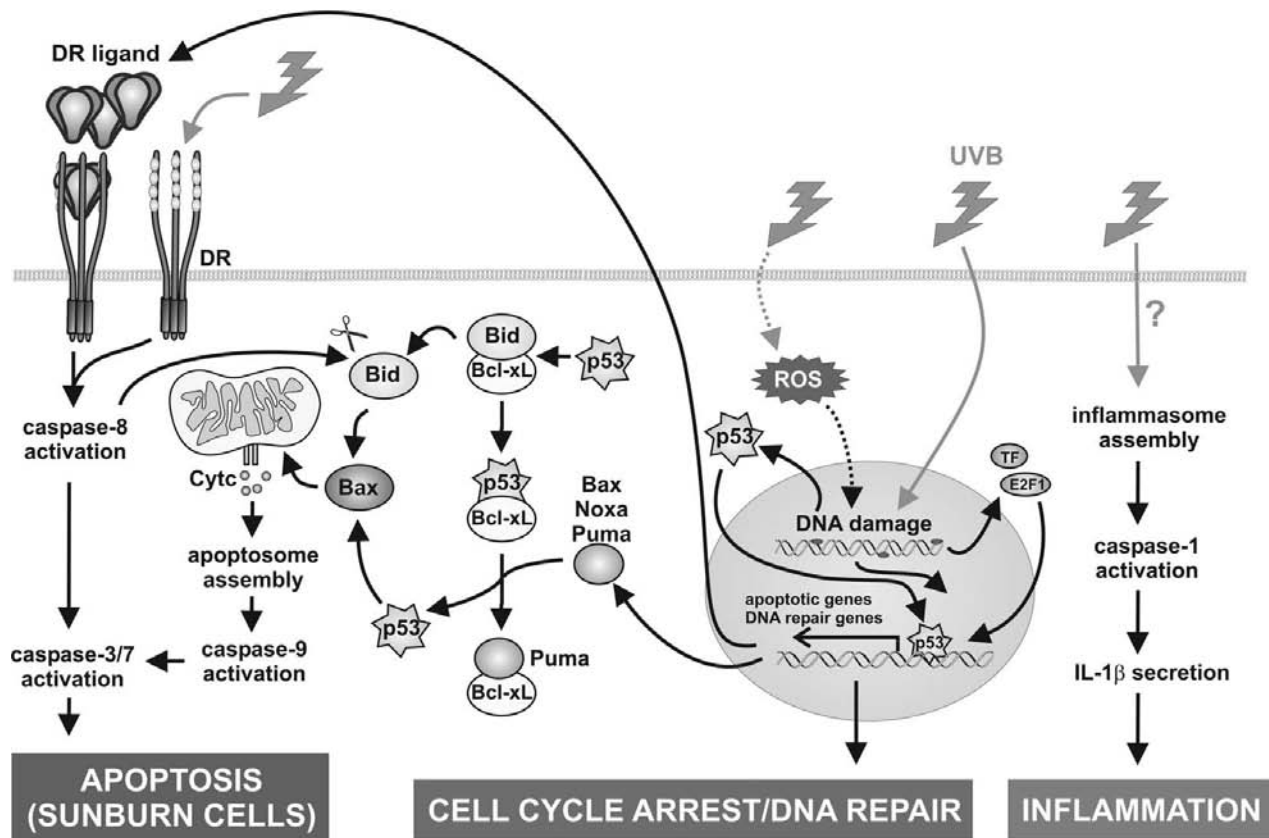


Figure 28-3. UVB signaling in keratinocytes. UVB can lead to different effects in keratinocytes, ranging from cell cycle arrest, apoptosis, and inflammasome activation. UVB radiation primarily damages nuclear DNA as a result of direct absorption and generates ROS that can induce oxidative damage to DNA and cellular proteins. The DNA damage is sensed by the DNA repair system. This leads to cytosolic stabilization of p53. In addition, other transcription factors (TF), such as NF- κ B and E2F1, become activated. In the first instance, these transcription factors will drive gene expression to repair the UVB-induced damage. E2F1 is a main transcription factor stimulating DNA repair genes in keratinocytes. The p53 transcription factor mediates either cell cycle arrest or apoptosis, depending on the extent of the damage. Sustained activation of p53, together with other transcription factors, will lead to the increased expression of the proapoptotic Bcl-2 family members Puma, Bax, and Noxa, which will lead to mitochondrial damage and subsequent cytochrome c release, and of death receptors and their ligands, which will activate extrinsic apoptotic pathway. Cell surface death receptors may also be activated directly in a ligand-independent way at high doses of UV radiation. This will lead to activation of the apoptotic caspase cascade. Caspase-8 activation can cleave the proapoptotic Bid molecule to enhance its activity. However, the extrinsic pathway does not substantially contribute to the overall cell death. Surprisingly, UVB-induced apoptosis does not require de novo protein synthesis, and p53 contributes to cell death, possibly through direct interaction with the mitochondrion itself and/or with members of the Bcl-2 family. A number of cytoplasmic and mitochondrial activities of p53 have been proposed. Bid can be chased from Bcl-X_L by competing p53 and induce Bax-dependent cytochrome c release. Alternatively, p53 can activate the expression of PUMA and other proapoptotic Bcl-2 family members, which then serve to release cytoplasmic p53 from the inhibitory interaction with Bcl-xL. The released p53 is then free to directly activate Bax. Bax will then induce cytochrome c release from the mitochondria. Many other signaling pathways and potential cross-talks were left out for reasons of clarity. Finally, UVB irradiation can also lead to inflammasome assembly and caspase-1 activation. However, the molecular mechanism leading to inflammasome activation is still unclear. See Color Plate 33.

3. CELL DEATH IN SKIN PATHOLOGY

3.1. Sunburn

Although CE formation does not occur through classical apoptosis, all components required for apoptosis are present in keratinocytes. One of the major environmen-

tal insults to which our skin is exposed is ultraviolet radiation (UVR), a potent agent that causes DNA damage and apoptotic sunburn cells. UV-induced apoptosis is a complex cellular process that involves many players, which are discussed next (Figure 28-3).

UVB (290–320 nm) can be directly absorbed by DNA, resulting in the formation of photo lesions such as

cyclobutane-pyrimidine dimers (CPDs) and pyrimidine (6–4) pyrimidone. UVA radiation (>320 nm) penetrates deeply into the skin, and the genotoxic effects are mainly mediated by accumulation of reactive oxygen species (ROS) generated by activation of photosensitizers (riboflavin, porphyrins, quinones). ROS production is also excessive in UVB-irradiated skin and contributes to apoptosis, as witnessed by the protective effect of antioxidants. Most of the generated photoproducts are removed by the cellular DNA excision repair. If not, permanent mutations can lead to cancer development. Upon excessive DNA damage, the cell will undergo apoptosis, resulting in the formation of so-called sunburn cells, characterized by the presence of photo lesions, pyknotic nuclei, and eosinophilic cytoplasm.

An important player in UVB-induced apoptosis is the transcription factor p53. p53 is involved in a plethora of cellular functions, such as cell cycle arrest and activation of apoptosis, and is generally considered a tumor suppressor. By activating apoptosis, p53 protects the organism against accumulation of damaged cells that may develop into cancerous cells. The choice between cellular responses depends on the type of cell and stress and action of p53 co-activators, such as p300, CREB-binding protein (CBP), and P300/CBP-associated factor (PCAF). As mentioned previously, UVB can induce severe DNA damage. Upon DNA damage, MDM2, a p53-binding ubiquitinating enzyme that targets p53 for degradation, is inactivated, and as a consequence, p53 becomes stabilized. The important role of p53 in UV-induced apoptosis is demonstrated by the significant reduction in sunburn cells in p53 knockout mice after UVB irradiation. p53 is able to induce apoptosis through transcription-dependent or -independent mechanisms and can occur through the extrinsic and intrinsic mitochondrial apoptotic pathway. Cytoplasmic p53 can bind directly to several antiapoptotic members of the Bcl-2 superfamily, such as Bcl-2 and Bcl-X_L, thereby neutralizing their antiapoptotic activity and acting itself as a proapoptotic protein. Nuclear p53 can transactivate proapoptotic Bcl-2 family members, such as Bax, p53-upregulated modulator of apoptosis (PUMA), Bik/Nbk, and Noxa. In Noxa-deficient animals, UV-induced keratinocyte apoptosis is suppressed, whereas this is not the case in PUMA-deficient mice. This is consistent with the observation that the UV-induced Noxa levels are far higher than the PUMA levels. Mouse embryonal fibroblasts deficient for both caspase-3 and -7 are also resistant to UVB-induced cell death. This may imply the existence of a positive feedback loop between pre- (e.g., Noxa) and postmitochondrial (caspases) signaling molecules required for UVB-induced cell death.

Among the transcriptional targets of p53, there are several genes involved in ROS generation, whereas other p53-dependent genes have an antioxidant function, suggesting a role for p53 in controlling ROS levels in homeostatic and pathogenic conditions. Interestingly, p53 also protects the skin by augmenting skin tanning upon UV irradiation. It was shown that p53 regulates induction of pro-opiomelanocortin-derived bioactive peptides such as α -melanocyte-stimulating hormone, adrenocorticotrophic hormone, and β -endorphin, which are key players in melanin production and secretion.

The DNA repair-promoting transcription factor E2F1, which is upregulated after DNA damage, is also involved in UV-induced apoptosis. In several cellular systems, the proapoptotic potential of E2F1 has been demonstrated. However, *in vivo* data suggest that the function of E2F1 in skin is mainly antiapoptotic. Its *in vivo* antiapoptotic potential is clear from mice over-expressing E2F1 in the skin. In accordance, E2F1-deficient mice exhibit a higher abundance of sunburn cells upon UVR. E2F1^{-/-} p53^{-/-} double-knockout mice exhibit the elevated UVB-induced apoptosis, which is seen in E2F1 single-knockout animals. This implies that E2F1 can function in a p53-independent way. Chronic exposure to UVR can induce inactivating mutations in the p53 gene and thereby hamper its potency to induce apoptosis, making cells more prone to malignant transformation.

Two other members of the p53 family, p63 and p73, are capable of transactivating p53 target genes and have been implicated in the regulation of apoptosis. Mice deficient for p63 have no epidermis or other squamous epithelia and lack epithelial appendages. There is some debate concerning the mechanism by which p63 exerts its function in skin. It has been thought to mainly play a role in stem cell proliferation; others claim a role in epidermal lineage commitment. Studying p63 in skin differentiation is complicated by the fact that the p63 gene is transcribed into 10 isoforms, either containing (p63TA isoforms) or lacking the transactivation domain (Δ Np63 isoforms). A mouse model over-expressing Δ Np63 α specifically in the epidermis showed a reduction in UVB-induced skin apoptosis, probably as a result of competing with p53-dependent signaling. Interestingly, it has been demonstrated that in human keratinocytes deficient in p53 and p63, there is impaired repair of CPDs. In these cells there was a marked decrease observed in the expression of DDB2 and XPC, which are key DNA damage-recognition proteins. p73-deficient mice show, in contrast to p53-deficient mice, no increased spontaneous tumorigenesis, but exhibit neurologic defects; no skin abnormalities have been described.

Involvement of the intrinsic pathway upon UVB-induced keratinocyte apoptosis has been shown by inhibitor studies targeting caspase-9. In accordance with this, it has been shown that over-expression of Bcl-2 renders keratinocytes resistant to UVB-induced apoptosis. Expression of Apaf-1 is increased upon UV irradiation of keratinocytes. Caspase cleavage of PKC δ is required for UVB-induced apoptosis. The generated PKC δ catalytic fragment translocates to the mitochondria, targets antiapoptotic Mcl-1 for degradation, and triggers redistribution and activation of Bax. Besides apoptotic caspases, UVR can also activate inflammatory caspases, which are activated in inflammasome complexes. UV-irradiated human keratinocytes have been shown to secrete interleukin-1 β , a caspase-1 substrate, in an inflammasome-dependent manner. This makes the skin an important component of innate immunity, which is logical, given the fact that keratinocytes are continuously exposed to environmental stressors. When mice deficient for caspase-1 are irradiated with UVB, the number of infiltrated neutrophils is strongly reduced as compared with that of wild-type mice. How UVB is able to induce assembly and activation of the inflammasome is currently unknown. In cultured keratinocytes, the activation of caspase-1 is dependent on an increase in cytoplasmic Ca²⁺ concentration. Interestingly, UV irradiation of human epidermal keratinocytes results in an increase in intracellular Ca²⁺ concentration.

The skin of caspase-14 deficient mice is highly sensitive to UVB irradiation, which is characterized by increased UVB-induced CPDs and consequent keratinocyte apoptosis. This is probably due to a reduced UV scavenging effect of the stratum corneum. It is of note that filaggrin degradation, which gives rise to NMFs and urocanic acid, a major absorber of UVB, is impaired in caspase-14 deficient mice. However, the exact mechanism by which caspase-14 protects against UVB-induced apoptosis in the skin remains to be determined. The implication of caspase-14 in UVB protection may be reflected in the fact that caspase-14 has thus far been found only in terrestrial mammals.

DRs are also involved in UV-induced apoptosis, and their triggering can occur in ligand-independent and ligand-dependent ways. Ligand-independent UVB-induced clustering of the DRs Fas and TNF-R1 has been described in human keratinocyte cultures. However, UVB irradiation can also induce upregulation of DRs and its ligands, such as Fas and FasL and TNF. In accordance with this, mice that are deficient for FasL or TNF-R1 show a reduction in the occurrence of sunburn cell formation.

3.2. Skin cancer

An imbalance toward too little apoptosis or too much cell survival in the epidermis can result in skin tumor formation. Epidermal tumors are divided into melanoma and nonmelanoma skin cancers (NMSCs). The majority of NMSCs (80%) are basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs), both arising from cutaneous keratinocytes. Apoptosis is a major cancer defense system in the skin, and generally, triggering or resensitizing of the apoptotic pathway is used in different anticancer treatments. Mutations in the p53 gene, induced by chemical or UVB exposure, are probably an early step during tumorigenesis in BCC and SCC. Mice deficient in p53 are very sensitive to UV-induced SCC formation, and skin-specific transgenes for the p53 regulator MDM2 inhibit UV induction of p53 and are more susceptible to chemical carcinogenesis.

In correlation with their elevated survival status, tumors often have an altered expression of anti- or proapoptotic proteins. Cancer treatment often aims to restore or invert this imbalance to make use of the apoptotic program to kill the tumor.

For instance, Fas levels are decreased in BCC and in melanomas, probably as a result of oncogenic Ras. Certain melanomas even have inactivating mutations in the death domain of Fas. Increase in Fas expression levels is accomplished by different anticancer drugs and cytokines. In addition, TRAIL-R activated cell death by antibodies or recombinant TRAIL appears promising as a cancer therapy. Interestingly, cancer cell lines, including melanomas, are sensitive to TRAIL killing, whereas nontransformed cells are not, therefore reducing the possible side effects of TRAIL therapy.

Tumors can sometimes escape from apoptosis because of increased levels of antiapoptotic proteins. First, higher levels of FLICE-like inhibitory protein (FLIP) allow melanomas to escape from DR-induced apoptosis by conventional T cells. Second, the inhibitor of apoptosis (IAP) family member survivin is not expressed in normal skin, but it is found in NMSCs. Third, tumor resistance to cytotoxic agents and radiotherapy is associated with increased expression of antiapoptotic Bcl-2 family members such as Bcl-X_L and Bcl-2. In certain melanomas, low or absence of sensitivity to TRAIL-induced apoptosis can be overcome by knocking down FLIP, survivin, Bcl-2, or IAPs. One strategy to lower Bcl-2 levels in tumors is the use of antisense oligonucleotides, called oblimersen. However, this approach failed in clinical trials for melanoma (and other cancers) treatment. A promising alternative are

synthetic BH3-only peptides or small organic molecules that interact with the hydrophobic cleft of antiapoptotic Bcl-2 family members and that, as such, are able to induce apoptosis. An alternative therapy making use of IAP inhibitors is currently under development.

Instead of decreased sensitivity to apoptosis, over-activation of survival pathways can also be the basis of tumorigenesis in the skin. Transgenic mice that over-express Akt in basal keratinocytes, which leads both to increased proliferation and decreased apoptosis, develop spontaneous tumors and show increased sensitivity to tissue plasminogen activator (TPA)-induced carcinogenesis. Other survival pathways, such as the MAPK pathway, which controls cell growth and differentiation, are also important in skin tumorigenesis. However, these pathways can result in oncogenic or tumor-suppressor effects, depending on the cellular status. For example, the activator protein-1 transcription factor can promote or suppress skin tumor formation depending on its sub-unit composition.

As discussed previously (see [Section 2](#), Cell Death in Skin Homeostasis), NF- κ B signaling plays a crucial role in skin homeostasis. The role of NF- κ B in skin tumorigenesis is diverse; both activation or inactivation can be advantageous for cancer development. The tumor-promoting role of NF- κ B is demonstrated in familial cylindromatosis. This disease is caused by loss-of-function mutations in cylindromatosis (CYLD), a deubiquitinase responsible for dampening of NF- κ B activation. Hence absence of CYLD will result in over-activation of NF- κ B. Similar to human cylindromatosis, mice lacking CYLD are more sensitive to TPA-induced tumor formation, underscoring the importance of NF- κ B activation in the development of certain types of skin cancer. In SCC, Ras activity is often increased as a result of mutations in the gene itself that render it constitutively active. Cell cycle arrest induced by oncogenic Ras can be overcome by blocking NF- κ B, as shown by I κ B α overexpression. So here NF- κ B performs tumor-suppressor activity.

3.3. Necrolysis

Toxic epidermal necrolysis (TEN; Lyell's syndrome) and Stevens-Johnson syndrome (SJS) are rare acute dermatological diseases defined by epidermal necrosis and mucosal erosions, with large areas of loss of contact between epidermis and dermis and massive keratinocyte apoptosis. In SJS and TEN, up to 10% and 30%, respectively, of skin surface can be detached. The main cause is a severe adverse drug reaction, or

in some cases, bacterial infection. The effector cells of the disease are thought to be drug-specific cytotoxic T cells, but the pathophysiologic mechanisms are largely unknown. So far, no specific treatment exists. However, FasL expression is increased in lesional skin keratinocytes of TEN patients. Interestingly, Fas-sensitive Jurkat cells undergo Fas-dependent apoptosis when incubated on skin cryosections of TEN patients. In addition, the therapeutic potential of intravenous administration of human intravenously collected immunoglobulins from healthy donors, also containing neutralizing anti-Fas antibodies, has been demonstrated in TEN patients. These results suggest an important role of the Fas signaling pathway in the development of TEN. SJS and TEN were long believed to belong to a spectrum of disorders that included erythema multiforme majus (EMM); however, now SJS and TEN are considered to be different diseases than EMM. In EMM, skin eruptions are mainly found on the extremities, whereas in TEN, blistering is widespread. In EMM, mucosal lesions may occur, but are mostly limited to the oral cavity, whereas in TEN, there are severe mucosal erosions. Finally, in TEN the mortality rate is much higher than in EMM.

3.4. Pemphigus

Pemphigus diseases are autoimmune cutaneous blistering disorders characterized by the presence of autoantibodies against structural proteins of the intracellular junctions.

Apoptotic keratinocytes are present in lesional tissue of pemphigus vulgaris (PV) patients. When PV immunoglobulin autoantibodies are added to keratinocytes in vitro, FasL is secreted, reduction of Bcl-2 is observed, and several apoptotic proteins are upregulated. Similar results were obtained after addition of an antibody against Fas Receptor to cultured keratinocytes. These data point to a possible involvement of the extrinsic apoptotic pathway in PV; however, it should be noted that this hypothesis has not been proven in vivo.

3.5. Eczema

The term *eczematous dermatitis* (eczema) comprises a group of inflammatory skin diseases, such as atopic dermatitis, and that are often characterized by the formation of vesicles associated with exudation. It has been shown that keratinocyte apoptosis is important for vesicle formation. The pathogenesis of eczema is T-cell mediated; secretion of interferon γ by these cells

promotes upregulation of Fas in keratinocytes and thereby sensitizes them toward apoptosis. In addition, several data suggest a role for Fas/FasL as a positive regulator of pathological skin inflammation. Therefore, the mechanism of Fas/FasL signaling was investigated in reconstructed human epidermis. Surprisingly, these experiments indicated that FasL rather induced inflammation in reconstructed epidermis; this in contrast to monolayer keratinocyte cultures, in which FasL induced apoptosis. Unexpectedly, the epidermal growth factor receptor-ERK axis was found to be involved in the transcriptional inflammatory responses to FasL in the epidermis.

3.6. Graft-versus-host disease

Graft-versus-host disease (GVHD) can occur after allogeneic bone marrow transplantation and is the consequence of tissue damage induced by cytotoxic T cells. Acute and chronic GVHD are mainly manifested in the skin (cutaneous GVHD), the liver, and the gastrointestinal tract. Acute GVHD results in high mortality rates. Keratinocytes in cutaneous GVHD die by apoptosis mediated by FasL presented by the lymphocytes and secreted TNF α . In mice it was shown that the use of FasL- and/or perforin-deficient T cells in transplantation experiments reduces mortality and manifestation of the cutaneous GVH reaction. Importantly, neutralizing both TNF α and FasL with antagonizing antibodies completely abrogates the disorder. Blocking Fas alone lowers mortality rates, as does TNF blocking. However, when bone marrow transplantation is used as a therapy, this approach to dampen GVHD cannot be used because the graft-versus-leukemia reaction is TNF-dependent.

4. CONCLUDING REMARKS AND PERSPECTIVES

The skin is situated at the critical junction between the host and the environment and is subject to a variety of potentially damaging agents, including microbial organisms, toxins, and gene-damaging radiation. The maintenance of homeostatic conditions is crucial for the epidermal function. From this overview, it should be clear that proper control of apoptosis and inflammation during epidermal differentiation is of major importance in keeping the architectural integrity in check. Although terminal keratinocyte differentiation is a textbook example of programmed cell death, the apoptotic signaling cascade does not seem to be involved in epidermal differentiation leading to cornification.

The epidermal cells, such as keratinocytes and melanocytes, are the only cells of the body that are directly exposed to noxious UV irradiation. Several molecules involved in apoptotic signaling are required to remove cells with excessive UV-induced DNA damage. If not properly removed, these cells may become cancerous. Some members of the TNF superfamily, such as the death receptors and their ligands, play an essential role during epidermal development and homeostasis. The major consequence of death receptor signaling in the epidermis is apoptosis, inflammation, and the induction of developmental cues. The deregulation of death receptor signaling, such as signaling to apoptosis or NF- κ B activation, is involved in the pathogenesis of certain cutaneous diseases. The loss of the ability of damaged cells to undergo apoptosis contributes to tumor development and progression, whereas excessive apoptosis can lead the development of necrolysis, GVHD, and possibly eczematous dermatitis. Interestingly, recent evidence suggests that Fas/FasL signaling can convert from apoptotic to inflammatory signaling in keratinocytes, depending on the cellular conditions. This implies that apoptosis could serve to restrict uncontrolled inflammation, even at the price of temporary tissue damage.

During the past decade, significant advances have been made in understanding cell death signaling in health and disease. This has led to the conceptualization and development of new therapeutic tools targeting these signaling pathways, some of which are already being used in the clinic or in clinical trials. In addition, a better understanding of the role of apoptosis in a number of cutaneous diseases will lead to the further development of improved treatment protocols for these pathologies.

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Apoptosis is essential in the generation and function of the immune system. Many of the millions of T and B cells that are produced daily in the primary lymphoid organs are destined to die after a sorting process that keeps only cells that meet strict selection criteria. Successful cells receive a survival signal and emigrate to the periphery, where they will become the many soldiers that protect the organism against viruses, bacteria, and other unfriendly agents. In response to infection or immunization, antigen-specific cells become activated and proliferate, and some differentiate into effector cells. When the infection battle is won, most of these cells are eliminated by apoptosis to prevent their accumulation and the potential problems that it would cause. Defects in the apoptotic process in the hematopoietic system can promote autoimmunity, whereas too much apoptosis promotes lymphopenia and immunodeficiency. This review focuses on the two main pathways of apoptosis and their specific roles during the development and the function of the main cell populations of the immune system.

1. TWO APOPTOTIC PATHWAYS CONVERGE IN CASPASE ACTIVATION

Execution of the apoptotic program is the result of the activation of a family of aspartate-specific cysteine proteases, called caspases. Caspases pre-exist in healthy cells as inactive zymogens. Depending on their structure and their mode of activation, they are classified as *initiator* (i.e., caspases-8, -9, -10) or *effector* (i.e., caspases-3, -6, -7) caspases. Initiator caspase zymogens contain long pro-domains that allow their recruitment to activation platforms where they autoactivate. They then cleave and activate effector caspases that are responsible for the destruction of essential cellular proteins and the ultimate demise of the cell.

Two different pathways lead to caspase activation (Figure 29-1). The extrinsic pathway, also called death receptor pathway, is triggered by the oligomerization of death receptors of the tumor necrosis factor receptor (TNF-R) family (e.g., Fas, TNF-R1, DR4, DR5) after ligation by their specific ligands (including FasL, TNF, TNF-related apoptosis-inducing ligand [TRAIL]).¹ Death receptors contain an intracellular death domain (DD) responsible for the recruitment of the adaptor protein Fas-associated death domain (FADD) by DD homophilic interaction. FADD in turn recruits the initiator caspase-8 (and -10 in humans) through death effector domain (DED) interaction in a complex named death-inducing signaling complex (DISC), in which caspase-8 is activated by cleavage. Death receptor signaling can be inhibited by cellular FADD-like interleukin-1 beta-converting enzyme (FLICE) inhibitory proteins (cFLIP), which can be recruited to the DISC and block the activation of caspase-8.

Activation of caspase-8 triggers the caspase cascade leading to apoptosis. In type I cells, such as lymphocytes, the death-receptor pathway does not require the Bcl-2-regulated pathway, and initiator caspases-8 and -10 activate the effector caspases directly.² Genetic alteration of FADD has shown that these proteins are essential for death receptor-mediated apoptosis, but loss of either of these proteins does not affect sensitivity of T cells to a variety of death stimuli, including cytokine deprivation and cytotoxic stress.^{3,4,5} By contrast, in type II cells, such as hepatocytes, the death-receptor pathway and the Bcl-2-regulated pathway appear to cooperate to kill cells in response to death receptor activation. In this instance, the Bcl-2 family member Bid is activated by cleavage by caspase-8 and triggers mitochondrial events that amplify the initial signal through death receptors.⁶

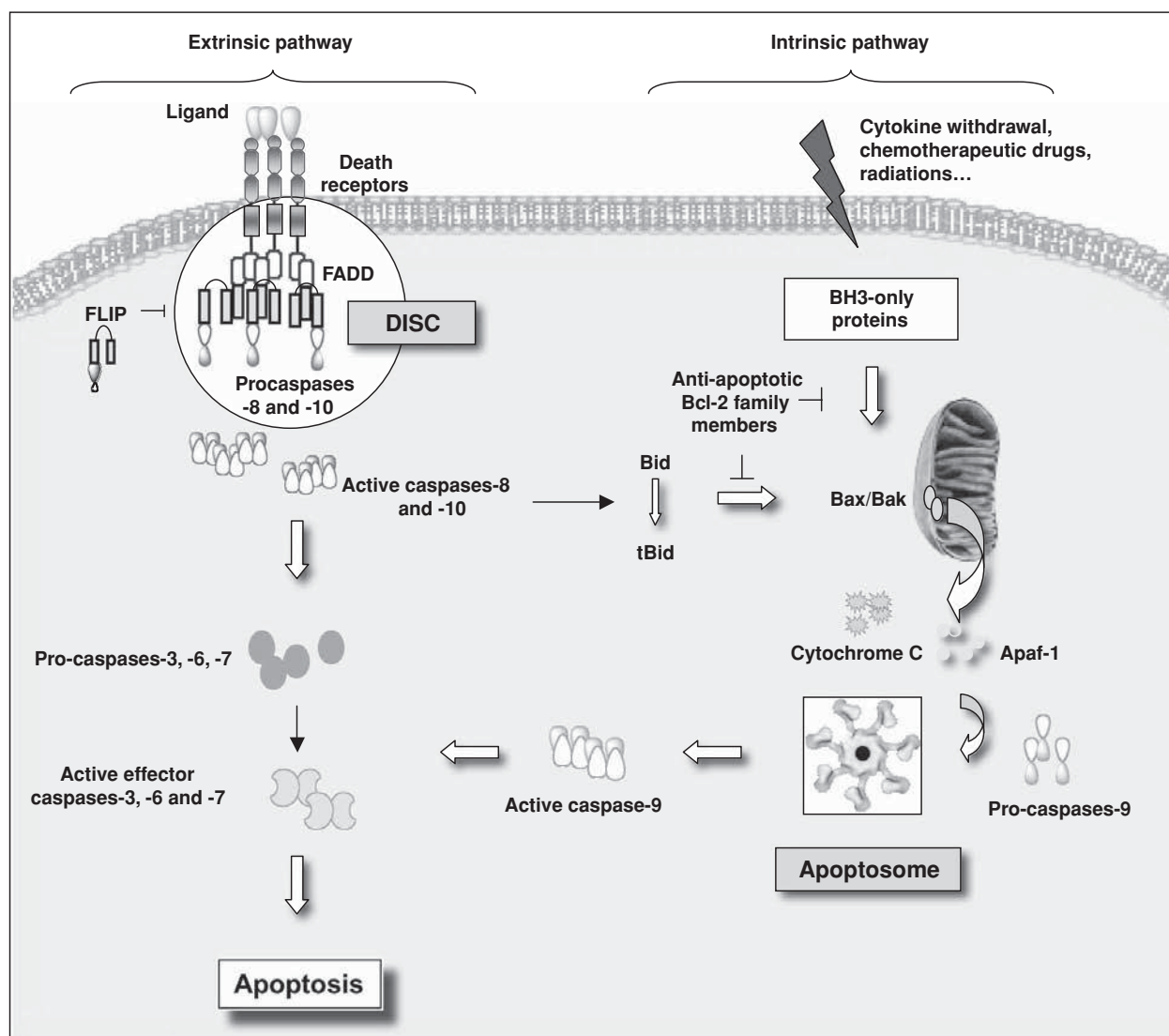


Figure 29-1. Two signaling pathways leading to apoptosis. The extrinsic pathway is triggered by the aggregation of death receptors upon ligand stimulation. This results in the recruitment of FADD and initiator caspases within the DISC. After activation, caspases-8 and -10 either directly cleave effector caspases (in type I cells) or induce the mitochondrial amplification loop through the cleavage of Bid (in type II cells). BH3-only proteins are activated in response to stress signals and trigger mitochondrial events that lead to the formation of a multi-molecular platform named apoptosome, in which the initiator caspase-9 is activated. This also results in the activation of effector caspases, responsible for the cell dismantling. See Color Plate 34.

Members of the Bcl-2 family are crucial regulators of apoptosis. The Bcl-2 regulated pathway (also called intrinsic, or mitochondrial pathway) requires the involvement of mitochondria (Figure 29-1).^{7,8} Proteins of the Bcl-2 family all contain 1–4 regions of homology, called Bcl-2 homology (BH) domains. According to the function and the structure of its members, the Bcl-2 family can be subdivided into three groups. The pro-survival members (Bcl-2, Bcl-xL, Bcl-w, A1, Mcl-1, and BOO) contain three or four BH domains. When over-expressed, they confer to the cells a resistance to various stimuli, such as cytokine withdrawal or cytotoxic stress,

including γ -radiations or chemotherapeutic drugs. The proapoptotic BH3-only proteins (Bad, Bik, Bid, Hrk, Bim, Noxa, Puma, and Bmf) act as sensors of cellular stress. They bind with high affinity to (at least some) prosurvival members and trigger apoptosis when over-expressed. Bim and Puma are potent cell death activators, capable of binding all of the prosurvival proteins. BH3-only proteins with a more limited binding repertoire such as Bad (which binds Bcl-2, Bcl-xL, and Bcl-w, but not Mcl-1 or A1) or Noxa (which binds only A1 and Mcl-1) appear to be weaker apoptotic inducers than Bim or Puma.⁷ The multidomain proapoptotic proteins (Bax, Bak, and

maybe Bok) contain two or three BH domains and trigger apoptosis when over-expressed. They act downstream of BH3 only proteins, because Bax^{-/-} Bak^{-/-} cells are resistant to BH3-only protein over-expression. In healthy cells, Bax is found in the cytoplasm, but it gets activated and moves to mitochondria on induction of apoptosis. Bak always localizes to the mitochondrial outer membrane, but it also becomes activated on apoptosis induction.⁹ Activation of Bax and/or Bak results in their oligomerization and mitochondrial outer membrane permeabilization (MOMP). This allows the release of apoptogenic proteins (in particular, cytochrome c) from the mitochondrial intermembrane space. In the cytosol, cytochrome c associates with apoptosis protease activating factor 1 (APAF-1) and the initiator caspase-9 to form the apoptosome, which triggers the autoactivation of caspase-9 and starts the cascade of effector caspases.

How exactly the proteins of the Bcl-2 family induce MOMP is the subject of an intense debate (for review see references 7, 8, 9).

Genetic experiments involving gene ablation or transgenic expression of many Bcl-2 family members, as well as proteins of the death receptor pathway, have helped define the role of these two major apoptotic pathways in the homeostasis and function of the immune system. Their role in the multiple maturation steps of T and B cells in particular is discussed.

2. APOPTOSIS AND SURVIVAL IN THE DEVELOPMENT AND HOMEOSTASIS OF THE IMMUNE SYSTEM

Apoptosis is essential for the development of all cell lineages but plays a very particular role in the immune system. Throughout most of adult life, lymphocyte numbers remain constant, as a result of a fine balance between proliferation and apoptosis.^{10,11} Cells of the immune system are generated from progenitors residing in the bone marrow. Most of these cells have to undergo several developmental stages to become functional immune cells. For instance, the survival of lymphocyte precursors is mediated by cytokines, which regulate the number of progenitor cells and initiate the rearrangement of the antigen receptor genes. B- and T-cell maturation involves the production by these cells of a functional antigen receptor (BCR or TCR, respectively). Cells that fail this process do not receive a survival signal from the receptor and die by apoptosis. The resulting pre-TCRs or pre-BCRs signal survival of progenitors and initiate their further differentiation. The processes of positive and negative selection then ensure that the interaction between the functional receptor and the

major histocompatibility complex (MHC) is adequate. All the lymphocytes whose receptors do not fit the selection criteria are eliminated by apoptosis.

The last important role of programmed cell death in the immune system is the elimination of responding lymphocytes at the end of an immune response. This mechanism of immune homeostasis is essential to prevent the nonspecific tissue damage that prolonged immune responses could cause and limit the risk of autoimmunity.

2.1. Survival of early hematopoietic progenitors

The hematopoietic stem cells (HSCs) give rise to multipotent progenitors (MPPs), which in turn give rise to the common lymphoid progenitors (CLPs) and the common myeloid progenitors (CMPs). CMPs give rise to the erythroid, megakaryocytic, granulocytic and monocytic lineages (Figure 29-2). In the present chapter, we focus on immune system development, without considering the erythroid and megakaryocytic lineage.

Because none of the differentiated lineages have self-renewal capacity and most have a limited life span (e.g., neutrophils have a life span of 6–18 hours), there is a constant requirement for the production of new cells from the bone marrow to ensure the turnover of the differentiated cells. The role of the Bcl-2 family members in the different developmental stages of B and T cells has been defined more precisely in the last 5 years.

Ectopic expression of Bcl-2 increases the number of hematopoietic progenitor populations and increases their ability to repopulate irradiated hosts.^{12,13,14} These cells are also protected from several death stimuli.¹⁵ These observations suggest that some proapoptotic BH3-only proteins are involved in the killing of progenitors, but do not prove that Bcl-2 is the main pro-survival member of the family involved in this process. Indeed, loss-of-function studies have demonstrated that mice deficient for Bcl-2 could still generate all blood lineages,^{16,17,18,19} showing that other prosurvival proteins are involved in the protection of HSC.

Similarly, Bcl-xL-deficient embryonic stem (ES) cells could also give rise to mature T cells, but not mature B cells.^{20,21} Bcl-xL expression increases dramatically when T cells differentiate from CD4⁻CD8⁻ (double negative, DN) thymocytes to CD4⁺CD8⁺ (double positive, DP) thymocytes. In contrast, single-positive (SP) thymocytes express negligible amounts of Bcl-xL protein. This expression pattern differs from that of Bcl-2, which is present in DN thymocytes, downregulated in DP thymocytes, and re-induced upon maturation to SP thymocytes.²¹

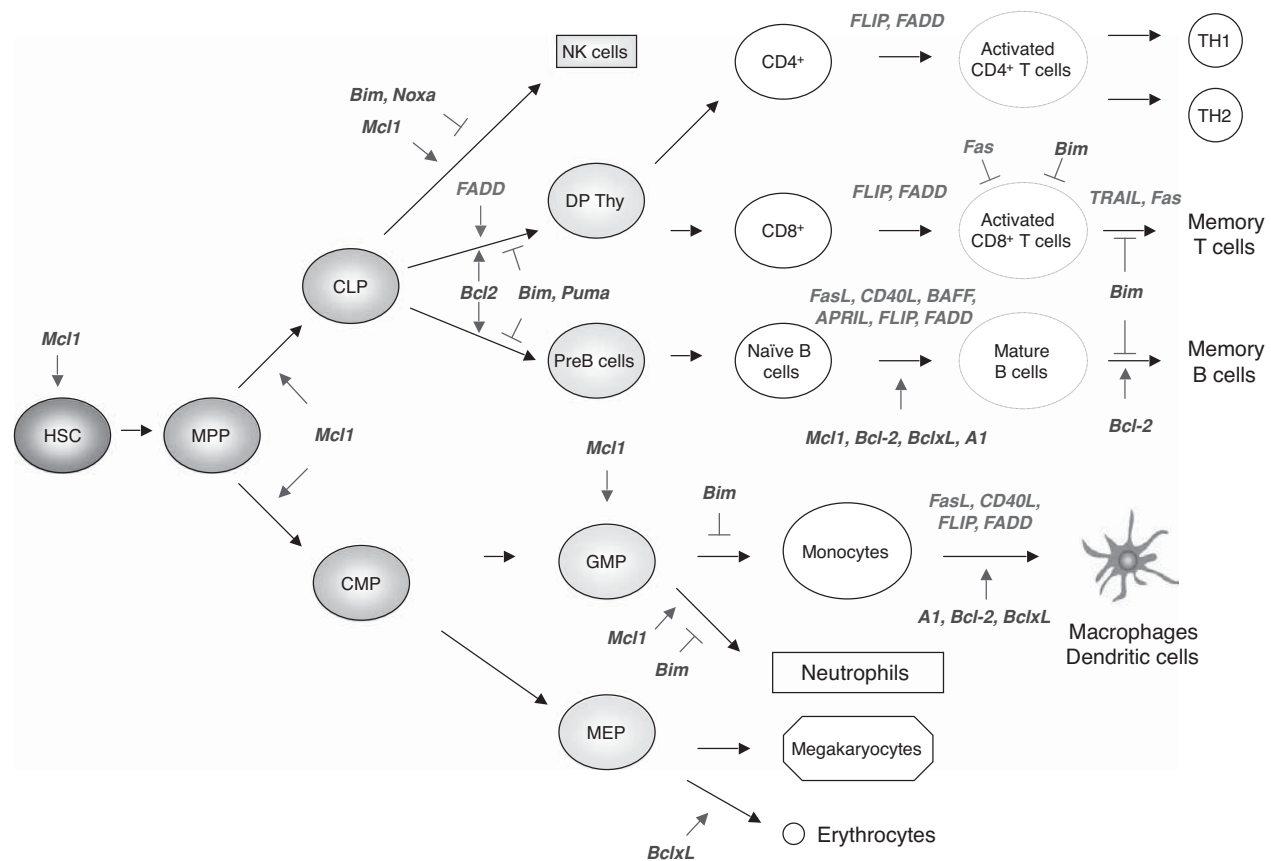


Figure 29-2. Involvement of Bcl-2 and TNF family members in hematopoiesis. The multiple steps of hematopoiesis are controlled by proteins of both the intrinsic and extrinsic pathways (see text for details). Their implication in various immune cell lineages has been mainly documented by gene deletion and transgenesis studies in mice. Mcl-1, Bcl-2, and Bcl-xL are critical for the survival of all cell lineages, but Mcl-1 seems to intervene more in the survival of early progenitors. These prosurvival proteins counteract the cytotoxicity of BH3-only proteins (mostly Bim, Puma, and to a lesser extent, Noxa). Bim in particular plays a crucial role in the sizing of both lymphoid and myeloid lineages. Death receptors can act independently or synergize with Bcl-2 family members.

Bcl-w RNA is detectable in most myeloid and some lymphoid cell lines, but loss of Bcl-w had no effect on the numbers of bone marrow HSCs.²²

The role of A1 in the immune system has been poorly characterized, mainly because of the fact that three A1 genes exist in the genome and that inactivation of the three genes at the same time has not been possible. Downregulation of the expression of the three genes may now be possible through the use of transgenic RNA interference.

By contrast, Mcl-1 has been described as an essential prosurvival protein during early hematopoiesis. The high level of Mcl-1 expression in long-term HSC and its subsequent decline in MPP, CLP, and CMP suggest that Mcl-1 may play a pivotal role in the earliest stages of hematopoiesis.²³ Interestingly, deletion of Mcl-1 in early hematopoietic development results in a rapid, fatal, and multilineage hematopoietic failure.²⁴ Later in myeloid development, Mcl-1 exhibits a selective role

being required for the terminal stages of granulocyte development but is dispensable for monocytic differentiation.²⁵ BH3-only proteins Bim, Puma, and Noxa have been shown to interact with Mcl-1 and thus are potential candidates for the downsizing of early progenitor populations.²⁶ Bim-, Puma-, and Noxa-deficient mice do not exhibit abnormalities in the resting hematopoietic progenitor populations,^{27,28,29} suggesting that none of them has an exclusive role in the determination of HSC numbers.

Consistent with the observation that Bcl-2 overexpression protects cells from cytokine withdrawal or ionomycin-induced apoptosis, Bcl-2 and Bim proteins display critical opposing roles in controlling lymphocyte homeostasis. Importantly, it has been described that the removal of Bim is able to rescue defects observed in Bcl-2^{-/-} mice.³⁰ Whereas a deficiency in Bcl-2 reduced the number of lymphoid and myeloid cells, the loss of one allele of *Bim* ameliorated this deficit and loss of both

Bim alleles more than restored normal number, suggesting that other prosurvival proteins, such as Mcl-1³¹ or Bcl-xL,²¹ are involved as guardians of Bim toxicity. Because maintenance of homeostasis appears to require a delicate balance between BH3-only proteins and their prosurvival counterparts, it would be interesting to know whether loss of Bim alone is sufficient to compensate for Mcl-1 deficiency in hematopoietic progenitor cells. Puma and Noxa, also able to bind Mcl-1, are also important regulators of lymphoid and myeloid cell apoptosis.^{28,29}

2.2. Sizing of the T-cell population

2.2.1. Establishing central tolerance

T-cell progenitors emigrate to the thymus from the bone marrow. Their maturation in the thymus consists of several steps, the purpose of which is the production of a functional TCR. Early T-cell progenitors are CD4⁺8[−] and can be subdivided into four populations according to expression of CD25 (interleukin [IL] 2 receptor α) and CD44. Pro-T1 cells (CD25⁺44⁺) are the most immature and develop successively into pro-T2 (CD25⁺44⁺), pro-T3 (CD25⁺44[−]), and pro-T4 (CD25[−]44[−]) cells.³² Signals through the IL-7 receptor (IL-7R)/ γ c, c-Kit (SCF receptor), and Flk2 are essential for cell proliferation and survival during the pro-T1 to pro-T3 stages of development.³³ Rearrangement of TCR β genes occurs during the transition from the pro-T2 to the pro-T3 stage.³⁴ Successful production of a pre-TCR³⁵ results in progression to the pro-T4 and CD4⁺8⁺ pre-T stages.³³ Thymocytes that survive the pre-TCR checkpoint proliferate and differentiate to yield the CD4⁺8⁺ population. The next maturation step is the rearrangement of the TCR α gene, and thymocytes expressing a complete TCR α / β -CD3 complex become subject to immunological selection on the basis of their TCR α / β specificity.³⁶

All in all, approximately 90% of the pro-thymocytes that enter the thymus will fail the selection process and undergo programmed cell death.³⁷ Immature T cells that do not produce a TCR and those whose TCR is not functional die “by neglect”. T cells that produce a functional TCR are then sorted according to the affinity of this receptor for a particular class II–self-peptide complex, in the process of positive and negative selection. T cells that recognize such complexes with moderate affinity are positively selected and differentiate into CD4⁺8[−] or CD4⁺8⁺ SP thymocytes before escaping to the periphery. The purpose of negative selection is to eliminate T cells whose TCRs have a high affinity for self-antigens and whose transformation into mature T cells and

subsequent escape to the periphery might cause autoimmunity. Such cells, however, sometimes reach the periphery, where they can be induced to die by mechanisms ensuring peripheral tolerance (see Section 2.2.2).

Early T-cell progenitors require the continuous presence of IL-7 for survival (pro-T1 to pro-T3 stages). Mice that lack IL-7, IL-7 receptor alpha (IL-7R α), or the common gamma chain (γ c), which is a component of the receptors for IL-2, -4, -7, -9, -15, and -21, have dramatically reduced numbers of T and B cells.^{38,39} IL-7–induced survival involves proteins of the Bcl-2 family, because the over-expression of Bcl-2 in IL-7R α -deficient mice restores T-lymphocyte numbers similar to those of wild-type mice.^{40,41} In vivo, the protection of early thymocyte progenitors by IL-7 seems to be ensured by Mcl-1 rather than Bcl-2 or Bcl-xL. Indeed, genetic models have demonstrated that neither Bcl-2 nor Bcl-xL are required for early lymphoid development,^{16,18,20,42} whereas mice deficient for Mcl-1 present an increase in apoptosis before antigen-receptor rearrangement strikingly similar to the phenotype of IL-7 or IL-7R α knockout mice.³¹ The nature of the proapoptotic proteins involved in early lymphoid development is still unclear. Experiments in mice lacking Bad, Bid, Hrk, Blk, or Noxa have shown that these BH3-only proteins are not crucial for early lymphoid development.¹¹ Bim is probably involved in the killing of immature thymocytes in the absence of IL-7 signaling because removal of Bim rescued near-normal numbers of mature T cells in *IL-7R α ^{−/−}* mice.⁴³ Because the rescue was not as complete as the rescue afforded by Bcl-2 over-expression, it is highly probable that other BH3-only proteins may be involved in the killing of T cells in the absence of IL-7 signal. Puma is a good candidate for such an activity, because loss of Puma in cultured myeloid cells rendered the cells resistant to growth factor withdrawal,²⁹ and loss of both Bim and Puma additively increased resistance to cytokine withdrawal,⁴⁴ suggesting that they act in concert in this killing activity.

Pro-thymocytes must rearrange their TCR genes to produce a functional MHC-restricted TCR. The first step in the production of a TCR is the rearrangement of the genes coding for the TCR β chain, which in combination with the invariant chain pT α and the CD3 protein, forms the pre-TCR. Successful assembly of a pre-TCR complex constitutes the pre-TCR checkpoint and is absolutely required for further differentiation. Indeed, mice lacking either of the recombination-activating genes, *Rag-1* or *Rag-2*, are defective in antigen receptor gene rearrangement and the development of their thymocytes is blocked at the pro-T3 stage.^{45,46,47,48} Lack of CD3 or pT α also blocks T-cell development at the pro-T3 stage.^{49,50} Over-expression of the antiapoptotic protein Bcl-2

rescues pro-T cells from a lack of IL-7R signaling,^{40,41} but it does not promote survival of pre-TCR-deficient pro-T3 cells in *scid*⁵¹ or *Rag-1*^{-/-} mice.⁴⁰ By contrast, expression of a dominant-negative form of the adaptor protein FADD, FADD-DN, restored significant CD4⁺8⁺ pre-T-cell numbers in *Rag-1*^{-/-} animals, demonstrating a role for the death receptor pathway in the apoptosis occurring in the absence of pre-TCR signaling.⁵² Fas has no role in the process, however, because T-cell development in Fas-deficient *Fas^{lpr/lpr}/Rag-1*^{-/-} mice was still arrested at the CD25⁺44⁻ pro-T3 stage.⁵² Assembly of a complete pre-TCR signals survival and proliferation of the pro-T4 population, as well as the next step in the production of the functional TCR, rearrangement of the TCR α gene. Bcl-xL has been proposed to protect DN and DP pre-T cells during TCR α gene rearrangement.⁵³ More recently, the prosurvival Bcl-2 family member A1 was identified as a direct transcriptional target of both pre-TCR and nuclear factor kappa B (NF- κ B).⁵⁴ A1 could be more important than Bcl-2 or Bcl-xL in this step, because mRNA knockdown of A1 impaired the survival of pre-T-cell lines despite the expression of the other prosurvival proteins. Further investigations will be necessary to understand whether the presence of any death receptor is required at this stage and how A1 can antagonize this apoptotic pathway.

Rearrangement of the TCR α gene results either in a nonfunctional α chain or in a chain that can be part of a TCR α / β /CD3 antigen receptor complex exposed on the surface of the pre-T cells. These immature thymocytes travel from the thymic medulla to the cortex and back and on their way meet thymic stromal antigen-presenting cells (APCs), which present on their surface self-peptides associated with MHC molecules. The fate of developing thymocytes is determined by the affinity of their newly assembled TCR for self-peptide-MHC ligands. Progenitors whose TCR has little or no affinity fail to receive a survival signal and undergo "death by neglect," a process in which Bcl-2 family proteins play an active role. Indeed, over-expression of Bcl-2 inhibits this type of death,⁵⁵ but over-expression of FADD-DN does not.⁵⁶ Even if Bcl-2 over-expression impairs cell death of thymocytes bearing TCRs unable to bind MHC molecules,^{51,55} their differentiation is arrested at the CD4⁺CD8⁺ stage, indicating that TCR-ligation activates signals required not only for cell survival, but also for differentiation.^{51,57} Bim is a likely candidate to mediate death by neglect because Bim deficiency confers resistance to cytokine withdrawal-induced cell death to DP thymocytes.²⁷ This, however, has not been formally demonstrated in a mouse model, and other BH3-only proteins may be involved in this process.

A TCR with intermediate affinity for self-peptide-MHC ligands mediates positive selection, and cells with such a TCR receive a survival signal, upregulate Bcl-2,⁵⁸ and differentiate into SP mature T cells that emigrate to the periphery. By contrast, immature thymocytes that harbor a high-affinity TCR are normally deleted by apoptosis in a process referred to as negative selection. This process seems to happen independently of the death receptor pathway because mice lacking Fas⁵⁹ or caspase-8⁵ or mice over-expressing FADD-DN⁵⁶ show no impairment in the death of autoreactive T cells. Negative selection has been reported to be partially impaired in TRAIL-deficient mice and in the presence of blocking soluble TRAIL receptor (DR5),^{60,61} but this result has been contradicted.^{62,63}

In the Bcl-2 family, Bim was shown to have a prominent role in the process of negative selection. Bim-deficient mice have a two- to four-fold increase in their numbers of single positive thymocytes and peripheral mature T cells.²⁷ *Bim*^{-/-} DP thymocytes were almost completely resistant to anti-CD3 antibody stimulation, whereas their wild-type counterparts were extremely sensitive to this treatment.⁶⁴ In the transgenic TCR HY model,⁶⁵ loss of Bim prevented the death of DP thymocytes in male mice in which they are normally deleted. Loss of Bim also protected thymocytes against apoptosis induced by superantigens.⁶⁴ TCR ligation induces accumulation of Bim and its association with Bcl-xL.⁶⁴ It has been reported that Bim accumulation is mediated by protein kinase C and Ca²⁺-dependent transcriptional activation.⁶⁶ In *Rag-1*-deficient mice reconstituted with Bax/Bak doubly deficient hematopoietic cells, thymocyte development is disrupted, with an alteration in thymocytes subsets similar to that observed in *vav*-Bcl-2 transgenic and *Bim*^{-/-} mice. Characteristically, these mice show an increase in the DN and SP numbers and a decrease in the DP population.^{13,27,67,68} *Bax*^{-/-}/*Bak*^{-/-} thymocytes were resistant to both death by neglect and antigen receptor-induced apoptosis.⁶⁸ This is not really surprising because loss of both Bax and Bcl-2 prevents the mitochondrial damage that triggers the caspase activation regulated by the Bcl-2 family. Interestingly, thymocytes from mice in which the immune system has been reconstituted with Apaf-1 and caspase-9-deficient fetal liver cells underwent normal negative selection.^{69,70} Similarly, *Rag*-deficient mice reconstituted with fetal liver cells deficient for both caspases-3 and -7 developed thymi with normal distribution of the SP and DP populations, although DKO thymocytes were highly resistant to apoptosis mediated by the mitochondrial pathway, at least at a short 24-hour time point. This data shows that Apaf-1 and caspases -9, -3, and -7 are not

absolutely required for the death of negatively selected thymocytes. This does not mean, however, that they are not normally used in this death process in wild-type mice. Indeed, cells devoid of Apaf-1 or caspase-9, -3, or -7 still have all the machinery necessary to damage mitochondria and release the contents of the mitochondrial inter-membrane space. It is possible that this mitochondrial damage is sufficient to cause the death of these cells even if the downstream apoptotic machinery is not functional. And it is possible that this death might not be apoptotic in these mutant cells, but rather necrotic.⁷¹ Further studies with these mutant mice will be necessary to test these hypotheses.

Another surprising observation is that, although loss of Bim induces a defect in thymic negative selection, over-expression of Bcl-2 does not protect autoreactive cells as well as loss of Bim does.^{64,72} This suggests that Bim has a function that is not inhibited by Bcl-2, but the nature of this function has not been discovered yet.

Nur77, Nor-1, and Nurr1 are a family of nuclear orphan receptors implicated in the death of autoreactive cells. Expression of both Nur-77 and Nor-1 is rapidly induced in thymocytes upon TCR stimulation, and their upregulation triggers a massive cell death in mature T cells,^{73,74,75} a process that seems dependent on their transcriptional activity.⁷⁶ Deficiency in either Nur-77 or Nor-1 does not lead to any impairment of negative selection,^{77,78} but their inhibition by a dominant-negative mutant inhibited negative selection.^{74,79,80} Although both Bim and Nur-77 are upregulated upon TCR engagement, there is no evidence that Bim may be a direct target of Nur-77 transcriptional activity.⁸¹ The function of Bim is affected in many ways by phosphorylation, and mitogen-activated protein (MAP) kinases ERK1/2, p38, and c-Jun N-terminal kinase (JNK) have been shown to phosphorylate Bim on selected serine and threonine residues.^{82,83,84} Interestingly, these kinases were also shown to have a role in negative and/or positive selection.^{66,85,86,87} A mutation of BimEL (T112A) affecting its phosphorylation by JNK was recently shown to impair negative selection in vivo in the HY-TCR transgenic animal model.⁸⁸ The authors concluded that the rescue of autoreactive thymocytes was due to the fact that the T112A mutation of Bim significantly decreases its binding to Bcl-2. A recently described and nonconventional MAP kinase, MEK5-ERK5, was also recently shown to regulate the level of Nur77 family members by transcriptional activation⁸⁹ and participate in the apoptosis of developing thymocytes. No direct connection between Bim expression and ERK5 activity was uncovered in this study. Studies on the role of Bcl-2 in autoreactive T cells may still hold

some surprises, because recent reports have indicated that Nur77 can translocate to mitochondria and interact with Bcl-2 family members (Bcl-2, Bcl-B, or A1).^{90,91} Upon TCR activation in DP lymphocytes, Nur77 and Nor-1 were shown to translocate to mitochondria and render Bcl-2 proapoptotic by inducing a conformational change that exposes its BH3 domain.⁹² It is thus possible that Bim and Nur77 may belong to two distinct pathways that converge at the mitochondria.

2.2.2. Peripheral tolerance

Mature CD4⁺ and CD8⁺ T cells that emigrate to the periphery should not be able to recognize self-antigens. Some do, however, because not all self-antigens are expressed in the thymus at a sufficient level to induce central tolerance. Mechanisms of peripheral tolerance have evolved to prevent autoimmunity that could result from the presence of autoreactive T cells in the periphery. This has been studied in a model of antigen cross-presentation in which naive T cells from TCR transgenic mice (OVA-specific CD8⁺ T cells from OTI mice, which do not express the antigen) are transferred into mice that express the antigen (OVA) transgenically. In a first report, deletion of Fas appeared to protect autoreactive CD8⁺ T cells from peripheral deletion in this system,⁹³ but a subsequent study by the same group refuted this finding and found that transgenic expression of Bcl-2 as well as genetic deletion of Bim could prevent the death of these cells.⁹³

These results were corroborated recently in a similar study using the clone 4 TCR transgenic cell line, which is specific for an H-2 K^d-restricted peptide epitope of the influenza hemagglutinin (HA).⁹⁵ The adoptive transfer of clone 4 CD8⁺ T cell into InsHA mice, which express the viral HA on their pancreatic islet β cells, results in T-cell activation by cross-presenting APCs in the pancreatic lymph nodes.⁹⁶ After adoptive transfer, Ag-specific clone 4 T cells underwent deletion independently of extrinsic death receptors, including Fas, TNFR1, or TNFR2. This deletion, however, could be inhibited by over-expression of Bcl-2 or targeted deletion of Bim, thereby resulting in accumulation of activated clone 4 T cells. Over-expression of Bcl-2 in clone 4 T cells promoted the development of effector function and insulinitis, whereas *Bim*^{-/-} clone 4 cells were not auto-aggressive. This data showed that initiation of clone 4 T-cell apoptosis during the induction of peripheral tolerance to a cross-presented self-Ag occurs through a Bcl-2-sensitive and at least partially Bim-dependent mechanism. Additional experiments are required to determine whether another BH3-only protein such as Puma could

be a partner to Bim in the death of autoreactive CD8⁺ T cells in the periphery, as suggested.⁴⁴

By contrast, the mitochondrial pathway may have no role in the death of autoreactive CD4⁺ T cells. Bcl-2 over-expression failed in inhibiting autoreactive CD4⁺ T-cell deletion in transgenic mice after adoptive transfer, whereas deletion is significantly impaired in *Fas^{lpr/lpr}* or *gld* (FasL mutant) mice.⁹⁷ However, the nature of the pathway involved in this process may depend on the quantity, the nature, and the expression pattern of the self-antigen.

Whereas Bcl-xL prevails over Bcl-2 for the survival of immature DP thymocytes, Bcl-2 expression is required for the survival of mature naive T cells in the periphery. The increase of Bcl-2 in resting cells could result from IL-4, -6, and -7 stimulation,⁹⁸ as well as IL-2 stimulation after T-cell activation.^{99,100} If Bcl-2 expression is required for survival, it is not essential for cytokine-mediated proliferation, because Bcl-2 transgenic T cells are able to survive, but do not proliferate, for instance, in the absence of IL-2.¹⁰¹ The role of Bcl-2 in the survival of T cells in blood and peripheral lymphoid organs has been highlighted by the fact that Bcl-2-deficient mice present a deficit in mature T cells.^{16,18,42} This deficiency can be compensated by the concomitant loss of Bim, showing that Bim plays an essential role in the apoptosis of mature T cells.^{27,30}

In response to infection or immunization, T cells that express antigen-specific TCRs become activated and proliferate, and some differentiate into effector cells.¹⁰² Activated T cells then produce cytokines that help coordinate the immune response aimed at eliminating the pathogen. Clearance of the antigen is accompanied by the shutdown of T-cell immune responses and involves apoptosis of a large fraction of antigen-activated T cells. This prevents accumulation of no longer needed and potentially dangerous effector cells, thereby maintaining homeostasis and precluding immunopathology.

The relative contributions of the two distinct apoptotic pathways in the termination of T-cell immune responses has been a matter of controversy for a long time. FasL and Fas have been implicated because activation-induced cell death, an in vitro model in which mitogen-activated T-cell blasts are killed by TCR re-stimulation, which causes FasL upregulation, is inhibited by FasL or Fas inactivation.^{103,104,105} Moreover, clearance of staphylococcus enterotoxin B (SEB)-activated TCR V β 8⁺ T cells was reported to depend partially on Fas.^{106,107} The mitochondrial pathway has been implicated in immune response shutdown, because Bcl-2 over-expression,¹⁰⁸ loss of Bim,^{43,109} or Bax and Bak deficiency⁶⁸ inhibited death of T cells stimulated in vitro or

in vivo by a single dose of SEB or in vivo after infection with human herpes simplex virus (HSV-1). Involvement of the death receptor pathway in clonal contraction was supported by the accumulation, in mice as in humans defective for either Fas (*Fas^{lpr/lpr}*) or its ligand (FasL),^{110,111} of excess mature T and B cells as well as "unusual" $\alpha\beta$ TCR⁺CD4⁻CD8⁻B220⁺ T cells, resulting in progressive lymphadenopathy and splenomegaly. Involvement of the mitochondrial pathway in the same process was supported by the observations that over-expression of Bcl-2 or loss of Bim also led to the accumulation of mature T and B cells in the periphery.^{13,27} However, the "unusual" $\alpha\beta$ TCR⁺CD4⁻CD8⁻B220⁺ T cells were not observed in Bcl-2 transgenic and Bim-deficient animals, strongly suggesting that these cells are normally eliminated by a system relying on Fas/FasL interaction, that is, the death receptor pathway.

Part of the controversy was solved recently, as a result of the observation that mice lacking both Fas and Bim (*Fas^{lpr/lpr} Bim^{-/-}*) accumulate extraordinary amounts of mature T, B, and the trademark "unusual" $\alpha\beta$ TCR⁺CD4⁻CD8⁻B220⁺ T cells, resulting in spectacular lymphadenopathy, splenomegaly, and an autoimmune pathology that develops very early.^{112,113,114} Clonal contraction was found to depend only on Bim in a model of acute viral infection (HSV-1), but to be the result of a cooperation between Fas and Bim in a model of chronic viral infection (MHV-68).¹¹² It thus appears that the relative contribution of the two main apoptotic pathways may be determined by the nature of the immune response (acute vs. chronic). In acute immune responses, the drop in cytokine amounts after clearance of the pathogen or injected immunogen triggers apoptosis.^{102,109,115} A single administration of SEB, which is known to be eliminated quickly from the body,¹¹⁶ would therefore mimic an acute infection. In contrast, TCR re-stimulation of activated T cells in vitro or repeated administration of SEB to mice is more likely to imitate the repeated TCR activation that is thought to occur during chronic immune responses, such as persistent infections or stimulation with self-antigens.^{102,115} Cooperation of both pathways is particularly obvious when considering the $\alpha\beta$ TCR⁺CD4⁻CD8⁻B220⁺ T cells. These cells indeed exist because of the loss of a functional death receptor pathway, because they do not accumulate in *Bim^{-/-}* or *Bcl-2*-transgenic mice. But their accumulation in *Fas^{lpr/lpr} Bim^{-/-}* mice exceeding by far the amounts observed in mice lacking Fas-only clearly shows the importance of Bim to limit this cell population in "normal" circumstances. The catastrophic consequences of the failure to properly eliminate activated B and T cells at the end of an immune response are

particularly obvious in *Fas^{lpr/lpr}Bim^{-/-}* mice. It is thus not a big surprise that natural selection would call on both apoptotic pathways (and also other tricks such as anergy and negative regulation) to prevent a fatal outcome.

2.2.3. Memory T cells

At the end of an immune response, the majority of effector T cells die by apoptosis, leaving behind a long-lived population of memory T cells. Upon re-infection by the same pathogen, these cells proliferate strongly and undergo a rapid transition into effector cells.^{117,118} Memory T cells have long been considered to be generated from the pool of effector T cells that have responded to foreign antigens, and there is indeed evidence that some memory T cells have previously displayed effector functions.¹¹⁹ But memory T cells display many characteristics that are typical of naïve cells, and this has led to the hypothesis that they may be generated directly from naïve T cells without going through the effector state.¹²⁰ The importance of cytokines, in particular of IL-7 and IL-15, in the maintenance and homeostasis of memory T cells is well recognized.¹¹⁸ Expression of IL-7R α on T cells at the peak of the effector response to acute infection has been first correlated with the ability to survive the downsizing of immune response and progress to memory,¹²¹ but recent reports indicate that IL-7 signal is not sufficient for this process.^{122,123,124} IL-2 and some inflammatory signals such as IL-12 or type I interferons play an important role in the differentiation of memory cells.^{125,126,127}

The relative role of death receptor and mitochondrial pathways downstream of these cytokines in the maintenance of memory T cells is not clearly defined yet. The balance between Bim and Bcl-2 has been described as critical for the homeostasis of the memory T-cell population,¹⁹ and accumulation of memory T cells has also been observed in Bak/Bax-deficient mice.⁶⁸ Interestingly, memory T-cell numbers were largely increased in mice lacking both Fas and Bim compared with mice lacking either of them alone,¹¹³ suggesting that here again, cooperation between both pathways may regulate the homeostasis of this cell population.

2.3. Control of apoptosis in B-cell development

2.3.1. Early B-cell development

The development of B cells resembles that of T cells in many aspects. The purpose of B-cell maturation is to produce a membrane BCR that can recognize foreign

antigens and ignore self-antigens.¹²⁸ As is the case for TCR, production of a functional BCR first entails the rearrangement of genes encoding the different subunits of the pre-BCR, composed of the immunoglobulin heavy chain (Ig HC) with the $\lambda 5$ and Vpre-B surrogate light chains. This happens at the pro-B cell stage, and successful assembly of the pre-BCR allows cells to survive and progress to the pre-B cell stages, during which immunoglobulin light chain (Ig LC) gene is rearranged and combined with Ig HC to form a functional BCR. As for the TCR components, rearrangement of BCR HC and LC is ensured by the recombination activating gene products RAG-1 and RAG-2, and only some of the rearrangement events lead to complete HC or LC proteins. All nonproductive HC or LC rearrangements lead to incomplete BCRs, and the lack of signaling that ensues results in apoptosis. Indeed, B cells in *Rag-1*- or *Rag-2*-deficient mice do not progress past the pro-B cell stage.^{48,129} This cell death process involves the Bcl-2-regulated pathway because over-expression of Bcl-2 and Bcl-xL prevents the deletion of pro-B cells in *Rag*-deficient mice, unable to produce rearrangement of Ig gene segments.^{130,131} Like for T cells, signals from IL-7 are necessary for the development of early B-cell progenitors, and B-cell development is arrested at the pro-B-cell stage in mice lacking either IL-7³⁹ or its receptor.³⁸ Surprisingly, although Bcl-2 over-expression restores T-lymphocyte numbers similar to those of wild-type mice, it fails to produce the same effect on the B-cell population.^{40,41} Loss of Bim in IL-7R α -deficient animals also rescued T cells, but had a much lesser effect on B cells.^{132,133} The requirement for IL-7 signaling in pre-B-cell survival ceases at the immature B-cell stage, where a signal from the BCR and a second signal from a TNF family ligand called BAFF (or BLys), through one of its receptors, are necessary for further differentiation and maturation of B cells.^{134,135} BAFF deficiency was found to arrest B-cell maturation at the immature transitional type I stage, whereas transgenic expression of BAFF causes accumulation of immature and mature B cells and autoimmunity.¹³⁶ Current evidence shows that all known BAFF receptors are expressed on B cells at different levels depending on their maturation and/or activation state.¹³⁴ BAFF-R is the key receptor that triggers BAFF-mediated survival, as mice deficient in this receptor display a phenotype similar to that of BAFF-null mice.¹³⁷

Maturation stages beyond the immature B-cell stage and survival of mature B cells require continuous signaling through the BCR, as inducible deletion of Ig genes was shown to cause a rapid disappearance of B cells.¹³⁸

2.3.2. Deletion of autoreactive B cells

Like autoreactive T cells, B cells that express a BCR that recognizes self-antigen must be deleted (negative selection) to prevent autoimmunity. Such B cells may also undergo further receptor editing to reduce the affinity of the BCR for self¹³⁹ or become anergic.¹⁴⁰ Deletion of autoreactive B cells can occur in the bone marrow as well as in the spleen and can happen at several developmental stages, from immature pre-BCR bearing cells to mature B cells in germinal centers.^{141,142} BCR ligation-induced deletion of autoreactive B lymphocytes in vivo is independent of Fas^{143,144} and not inhibited by over-expression of FADD-DN or caspase-8 inhibitor CrmA in immature B cells lines.¹⁴⁵ However, this process can be inhibited by Bcl-2 or Bcl-xL over-expression.^{131,140,146} Of all BH3-only proteins, Bim again appears as the best candidate to signal death downstream of BCR engagement. Indeed, mice lacking Bim were shown to develop autoimmunity and express autoantibodies.²⁷ In the anti-HEL Ig/HEL model of autoreactive B-cell deletion, loss of Bim protected B cells against BCR-induced ligation, demonstrating its role in the process.¹⁴⁷

It is interesting to note that genetic background has a strong influence on the consequences of Bim deficiency. The original *Bim*^{-/-} mice were generated by a targeted mutation in 129Sv ES cells and backcrossed onto C57BL/6 background. Mice of the few first generations developed fatal autoimmune disease at high frequency, whereas homozygous mice produced after >20 generations of backcross have less severe symptoms.^{27,64} Similarly, BCR-induced apoptosis was shown to be deficient in mice of the MRL background.¹⁴⁵ It would be interesting to identify the genes responsible for these differences, as they may be modulators of the Bcl-2-regulated pathway.

2.3.3. Survival and death of activated B cells

Mature B cells critically depend on the BCR signal as well as on an auxiliary signal from the TNF family member BAFF for their survival.¹³⁴ Both signals are not independent, because BCR ligation upregulates BAFF receptor (BAFF-R) expression on B cells.¹⁴⁸ Although BAFF has three receptors (BAFF-R, TACI, and BCMA), only BAFF-R seems to be the key receptor that signals BAFF-mediated survival, as mice deficient for this receptor display the same phenotype (absence of peripheral B cells) as mice deficient for BAFF.^{149,150} By contrast, TACI seems to be a negative regulator of B-cell survival, as B-cell numbers are increased in TACI-deficient mice, and these animals eventually develop autoimmune disease.¹⁵¹ Survival

signaling through BAFF and BAFF-R involves the activation of the NF- κ B pathway, but the details of the signaling cascade have not been elucidated yet. In any case, BAFF has become a major focus of research since the confirmation of its involvement in rheumatoid arthritis and systemic lupus erythematosus-like autoimmune diseases. Several clinical trials using BAFF-neutralizing agents are currently under way.¹³⁴

The accumulation of antibody-forming cells (AFCs) and consequently abnormally high serum Ig levels in Bim-deficient mice demonstrated the role of Bim in the termination of B-cell immune responses.²⁷

Because Bcl-2 transgenic mice present an increased number of memory T cells,¹⁵² it has been postulated that Bim could be involved in the shaping of the memory compartment. A recent study showed that Bim-deficient mice accumulated large numbers of low-affinity Ig-expressing memory B cells, in addition to elevated numbers of AFCs.¹⁵³ Therefore, Bim does not interfere with the affinity maturation process, but seems to be critical for the removal of low-affinity antibody-bearing memory B cells. However, because Bcl-2 transgenic mice accumulated even more antigen-specific B cells than *Bim*^{-/-} mice, we cannot exclude that another BH3 only protein, such as Puma, could play a part in memory B-cell homeostasis.

The genetic studies mentioned previously involving deletion or over-expression of members of death receptor or Bcl-2-regulated pathways have highlighted that deregulation of cell death mechanisms in T and B lymphocytes have dramatic consequences, ranging from degenerative disorders to autoimmune pathologies. Excessive cell death due to the absence of survival signals (e.g., IL-7) or the lack of a prosurvival molecule (i.e., Bcl-2 or Mcl-1) can lead to a very fragile, if not completely absent, immune system. By contrast, insufficient cell death due to the absence of a proapoptotic mediator (FasL, Fas, Bim, etc.) can lead to an accumulation of immune cells that should normally die and culminate in a fatal autoimmune disease, such as glomerulonephritis. In the Bcl-2 family, Bcl-2 and Mcl-1 seem to be the most important prosurvival regulators. If we consider the expression of Bcl-2 family members in T and B cells,¹⁵⁴ it appears that the role of A1 might have been underestimated so far, probably because the genetic deletion of all three A1 genes at once presents a major challenge. Among the BH3-only proteins, Bim really stands out for its multiple roles in so many critical steps of T- and B-cell maturation. Bim plays a major role as a Bcl-2 inhibitor, as demonstrated by the fact that lack of Bim completely rescued the degenerative diseases caused by the lack of Bcl-2.³⁰ Puma also plays

a significant role in the homeostasis of the immune system, because concomitant loss of both Bim and Puma caused a hyperplasia of the lymphoid organ greater than that caused by loss of Bim alone.⁴⁴ Combined loss of Bim and Puma also promoted spontaneous lymphomagenesis, confirming the tumor suppressor potential of these two proteins.⁴⁴

3. IMPAIRED APOPTOSIS AND LEUKEMOGENESIS

Apoptosis serves as a barrier against oncogenic transformation. Resistance to apoptosis allows preneoplastic cells to acquire additional mutations and to grow in conditions that should normally signal their death, such as hypoxia.¹⁵⁵ Deregulation of apoptosis-related genes has been found in many types of human tumors.¹⁵⁶ Bcl-2 was initially identified because of its involvement in oncogenic chromosomal translocation in human follicular lymphoma.¹⁵⁷ On its own, *Bcl-2* proved to be a rather weak oncogene, but it was shown to dramatically accelerate *Myc*-induced lymphomagenesis.¹⁵⁸ *Myc* over-expression has been shown to signal proliferation as well as apoptosis, and Bcl-2 is thought to cooperate with *Myc* by antagonizing its proapoptotic effect.¹⁵⁹ Although *Myc* and *Bcl-2* synergize in tumor development, particularly lymphomagenesis, the initiation, development, continued growth, and severity of E μ -*Myc* lymphoma do not depend on endogenous Bcl-2.¹⁶⁰ Mice expressing an Mcl-1 transgene in hematolymphoid tissues were found to develop lymphoma with long latency and at high probability (>85% over 2 years). In most cases, the disease was widely disseminated and of clonal B-cell origin. A variety of histologic subtypes were seen, predominantly follicular lymphoma and diffuse large-cell lymphoma.¹⁶¹ Recently, Mcl-1 overexpression has also been shown to dramatically accelerate *Myc*-driven lymphomagenesis.¹⁶² Further investigations will be required to evaluate the role of endogenous Mcl-1 in this model. A1 has been reported to be required for leukemogenesis mediated by BCR/ABL,¹⁶³ and Bcl-xL has been implicated in mouse myeloid and T-cell leukemia.¹⁶⁴ Because antiapoptotic proteins of the Bcl-2 family displayed oncogenic potential, it was no surprise when Bim and Puma were found to be tumor suppressors.^{165,166} Similar to Bcl-2 over-expression, loss of Bim accelerated *Myc*-driven lymphomagenesis.¹⁶⁵ Significantly, mutations inactivating the Bim gene were also described in human mantle cell lymphomas and many Burkitt's lymphomas.^{167,168} Silencing of Bim in these tumors was achieved by gene deletion or promoter methylation.¹⁶⁸ Downregulation of Puma with shRNAs promoted oncogenic transformation of primary murine

embryonic fibroblasts by the E1A/Ras combination and accelerated *Myc*-induced lymphomagenesis.¹⁶⁶

Recent findings have indicated that Bim is regulated by the miR-17-92 microRNA cluster that is amplified in some human lymphomas.^{169,170}

From a therapeutic point of view, it is rather convenient that deregulation of the apoptotic machinery tends to an increase of the ratio of prosurvival molecules/ proapoptotic BH3-only molecules rather than a destruction of the machine itself (which could be achieved by the loss of Bax and Bak, for example). Many of the commonly used cytotoxic agents work by reversing the skewed ratio in favor of proapoptotic molecules (reviewed in reference 171). For example, the kinase inhibitor imatinib used to counteract the effects of the Bcr/Abl oncogenic protein in chronic myelogenous leukemia kills cells through Bim and Bad,^{172,173} whereas glucocorticoids used for the treatment of acute lymphocytic leukemia rely on Bim and Puma to kill cells.^{27,28,171} Because BH3-only proteins antagonize their prosurvival relatives by binding to them through their BH3 α -helical domain, the development of small molecules mimicking the BH3 domain has generated a lot of enthusiasm and hope in the last few years. Several such molecules have been described and are presently tested in the clinic (for review, see Zhang drug resistance updates).¹⁷⁴ Of particular interest, a small molecule developed by Abbott Laboratories using a structure-based approach, ABT-737, has been shown to have a very high affinity (nanomolar range) for Bcl-2, Bcl-xL, and Bcl-w.¹⁷⁵ ABT-737 proved very efficient at killing cells with low Mcl-1 content, whereas cells with high Mcl-1 content were resistant to the drug. Inhibiting Mcl-1 by over-expression of Noxa in these cells rendered them sensitive to ABT-737, showing that a BH3-mimetic with a limited binding spectrum might be used in combination with conventional drugs to kill cancer cells.¹⁷⁶ Understanding the subtleties in the relationships between Bcl-2 family members in the control of life and death in the immune system will be useful to design therapeutic regimes suited to each tumor type.

4. CONCLUSIONS

To become an active part of the immune system, hematopoietic stem cells have to undergo an educational program that will leave most of them dead before reaching their goal. This is the price to pay for the organism to avoid renegade cells taking over the system, leading to its ruin. The Bcl-2 regulated and death receptor pathways are in charge of the elimination of the cells that do not meet the stringent criteria of the system.

Although a lot has been learned about the different signaling mechanisms that decide cell fate at the different checkpoints, some pieces of the puzzle are still missing. How, for example, does a membrane receptor signal death or survival depending on the strength of its interaction for its ligand? There is still much to learn about the mechanisms that control life and death in the immune system, but the work that has been accomplished since the discovery of Bcl-2 has provided valuable insight into the workings of the life/death switches, and strategies based on this knowledge are beginning to emerge.

There is much hope that a better understanding of the mechanisms that orchestrate survival and cell death of immune cells will lead to the development of novel pharmaceutical strategies to prevent inflammation, autoimmune diseases, and cancer.

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1. INTRODUCTION

The hematopoietic system is a dynamic multilineage organ, from which we can gain insights into the physiologic roles of cell death pathways. The role of hematopoiesis is to produce blood cells under normal and stress conditions throughout the lifespan of an organism. In humans, effective function of the hematopoietic system entails the steady and regulated production of erythrocytes, which carry oxygen; platelets, which prevent bleeding; and granulocytes, monocytes, and lymphocytes, which are required for host defense. Remarkably, these varied cell types are derived from a common ancestor, the hematopoietic stem cell (HSC). HSCs are known for certain key properties: they are long-lived and self-renewing and give rise to a steady supply of multipotential progenitor cells (MPPs) that are committed to undergo differentiation. MPPs in turn give rise to progenitors with a progressively restricted developmental potential; these undergo regulated expansion and, eventually, commitment to terminal differentiation. The terminal differentiation program is characterized by decreased proliferation or mitotic arrest, global changes in gene expression, and cellular remodeling. Finally, cells that have fulfilled their purpose, or senescent cells, are cleared from the body to make room for new cells, or after a stress response to re-establish homeostasis.

As discussed in other chapters, cell death pathways can be divided, on the basis of their mechanism of action, into extrinsic and intrinsic arms. In a simplified view, extrinsic death pathways are activated by the binding of extracellular ligands to members of the death receptor family. These include Fas, tumor necrosis factor (TNF) family receptors, and TNF-related apoptosis-inducing ligand (TRAIL) family receptors.^{1,2,3} Activation

of death receptors leads to the assembly of a death-inducing signaling complex, which in turn leads to activation of apical caspases (caspase-8 in mice, caspase-8 and -10 in humans) and cleavage and subsequent activation of effector caspases (caspase-3 and -7). Activation of caspase-3 and -7 leads to cleavage of caspase targets, which include regulatory, structural, and homeostatic proteins, and to programmed cell death.⁴ Intrinsic death pathways are activated by cellular stress, such as DNA damage or growth factor deprivation, and these signal to mitochondria through the BCL2 family of proteins, causing cytochrome c release, assembly of the apoptosome, and activation of effector caspases.⁵

The BCL2 family of proteins can be divided into antiapoptotic and proapoptotic groups.⁶ Antiapoptotic BCL2-related proteins typically contain four BCL2-homology domains (BH1-BH4); these include BCL2, BCL-X_L, A1, BCL-W, and BOO. Proapoptotic BCL2-related proteins can be further subdivided into a multidomain proapoptotic group – BAX, BAK, and BOK – which contains three BCL2-homology domains (BH1-BH3), and a second larger group, which contains a single BCL2-homology domain (BH3-only proteins). In response to cellular stress, BH3-only proteins transduce proapoptotic signals to mitochondria where they directly or indirectly activate BAX or BAK,^{7,8} causing cytochrome c release and apoptosis.^{9,10} Additionally, a BH3-only protein, BID, can be cleaved and activated by caspase-8, linking the extrinsic and intrinsic pathways.^{11,12}

Distinct phases of hematopoietic development are under the control of cell death pathways; these include survival of stem cells, expansion and lineage determination of progenitor cells, terminal differentiation of precursor cells, function of formed blood cells, and the elimination of senescent cells. Therefore, the purposes of this

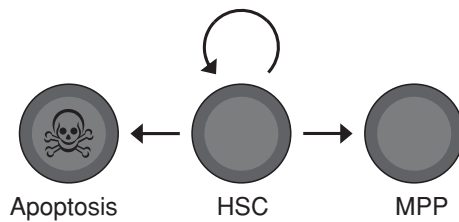


Figure 30-1. Hematopoietic stem cell fates. HSCs can either undergo self-renewal, differentiation to an MPP, or apoptosis. Depending on whether cell divisions are symmetric (give rise to identical progeny) or asymmetric (give rise to progeny with different fates), HSCs may increase in numbers, decrease, or stay the same.

chapter are (1) to review the roles of the extrinsic and intrinsic cell death pathways in hematopoietic development at each of these stages; and (2) to place these findings in the broader biological context of the regulation of cellular proliferation and differentiation.

2. HEMATOPOIETIC STEM CELLS

Long-term repopulating HSCs provide a steady supply of multipotential progenitor cells throughout the lifespan of an organism. Depletion of HSC leads to hematopoietic insufficiency and death. Therefore, HSC must be protected and the HSC pool maintained over an extended period of time. There are several potential cellular fates of HSC; these include self-renewal, proliferation, differentiation, and programmed cell death (Figure 30-1). These fates must be balanced to sustain a functioning HSC pool throughout the lifespan of an organism.

Chief among the mechanisms that preserve HSCs is their quiescent cell cycle status; the majority of HSCs are in the G0 phase of the cell cycle and undergo cell division about once per month.¹³ Infrequent cell division protects HSCs from cell cycle checkpoint-activated apoptosis; indeed, HSCs accumulate DNA damage over time, but their reserves are not significantly depleted, and a clear deficit in stem cell function only emerges under stress conditions, such as transplantation, when HSC are induced to divide.^{14,15,16} Another key mechanism for maintenance of the HSC pool is repression of differentiation potential. The HSC transcriptome overlaps that of other types of stem cells and includes genes that are important for self-renewal.^{17,18} When HSCs commit to differentiation, these genes are downregulated, and in a process known as multilineage priming, other genes characteristic of multiple hematopoietic lineages are expressed.¹⁹ Certain transcription factors and corepressors inhibit this fate change, and deficiencies of these factors are characterized by stem-cell depletion and other hematopoietic abnormalities.^{20,21,22,23}

The bone marrow microenvironment or niche is important for maintaining HSCs in a quiescent state and promoting HSC survival.²⁴ Stromal cells in the bone marrow microenvironment produce cytokines, which are implicated in HSC homeostasis; these include stem-cell factor, thrombopoietin, notch ligands, Wnts, and angiopoietin-1.²⁵ Among these, stem-cell factor, and additionally the growth factor Flt3 ligand, upregulate MCL1 in HSCs.^{26,27} MCL1 is highly expressed in HSCs and is essential for HSC survival.²⁶ On the other hand, BCL2 and BCL-X_L are dispensable.^{28,29} Overall, the role of apoptosis in HSC homeostasis was addressed through the use of Tg(*H2k-Bcl2*) mice, in which BCL2 expression is enforced by a constitutive promoter.³⁰ HSCs from these mice are expanded 2.4-fold as compared with wild-type controls and can out-compete wild-type HSCs in transplantation experiments. Thus inhibition of apoptosis is critical for HSC survival, but apoptosis has a limited role in the regulation of HSC numbers.

For the most part, the signal transduction pathways that regulate HSC homeostasis remain to be defined. One exception is Akt, which appears to play an essential role. Mutation of *Pten*, a negative regulator of Akt, leads to increased HSC cycling and depletion.³¹ Furthermore, the FoxO transcription factor family, which is negatively regulated by Akt, has a role in the maintenance of HSC quiescence through its inhibitory effect on reactive oxygen species (ROS) generation.³²

3. HEMATOPOIETIC PROGENITOR EXPANSION AND LINEAGE DETERMINATION

Regulation of HSC commitment to differentiation is not well understood, but may be related to disengagement of HSC from their specialized niche.²⁴ Once HSCs commit to differentiate, they start to cycle more frequently, lose their capacity for self-renewal, and develop into MPPs. The role of MPPs is to supply the proper number of differentiated hematopoietic cells to meet the immediate needs of the organism. To accomplish this, two processes are concurrently engaged and subject to feedback regulation: the first is regulation of cell numbers, and the second is lineage determination. With respect to the former, calculations of potential versus actual hematopoietic cell production show that a high percentage of early hematopoietic progenitors undergo cell death.³³ Thus MPPs and their progeny provide a significant reservoir of hematopoietic production.

Similar to HSC, MCL1 expression in hematopoietic progenitors is regulated by stem-cell factor, and MCL1 is essential for progenitor cell survival.²⁶ However, although Tg(*Vav-Bcl2*), *Bim*^{-/-}, and *Bax*^{-/-}; *Bak*^{-/-} mice

numbers of early and late erythroid progenitors, but are blocked at the post-CFU-E stage as a result of apoptosis. The EPOR is a member of the cytokine receptor family and lacks intrinsic tyrosine kinase activity; however, the EPOR membrane-proximal cytoplasmic domain is associated with janus kinase 2 (JAK2).⁴⁹ *Jak2*^{-/-} mice also die of severe anemia around E12.5 because of a severe defect of erythroid cell survival, demonstrating that JAK2 is essential for EPOR function.⁵⁰ BCL-X_L is highly expressed in erythroid cells,⁵¹ and it is suggested that BCL-X_L is a major downstream target of EPOR signaling^{52,53}; indeed, BCL-X_L is essential for erythroid cell survival, and enforced expression of BCL-X_L can supplant the requirement for erythropoietin (EPO) at the final stages of erythroid differentiation.^{29,54} On the other hand, EPO supports the survival of BCL-X_L-deficient basophilic erythroblasts, and the death of BCL-X_L-deficient erythroblasts occurs at a late stage of maturation.^{55,56} Thus BCL-X_L appears not to be the primary target of EPOR and JAK2 signaling that supports erythroid cell survival.

EPO deprivation causes caspase activation and apoptosis.^{51,57,58} The fact that BCL-X_L can rescue the survival of EPO-deprived erythroid cells suggests that death in this setting is mediated at least in part through the intrinsic pathway.⁵⁴ On the other hand, mature erythroblasts express membrane-bound FASL, and immature erythroblasts are sensitive to FAS-mediated apoptosis through the extrinsic pathway.^{1,59} Because these cells are in direct contact in three-dimensional erythroblastic islands in vivo,⁶⁰ it has been suggested that erythropoiesis is negatively autoregulated (Figure 30-2).^{59,61} The pattern of TRAIL and TRAIL receptor expression is also consistent with this model.⁶² Results from mutant mouse strains, however, are inconclusive: FAS-FASL signaling-deficient *lpr/lpr* and *gld/gld* mice do not have an overt erythroid phenotype, but *Caspase8*^{-/-} and *Fadd*^{-/-} mice exhibit erythrocytosis.^{63,64} Thus additional studies are needed to elucidate the death pathway opposed by EPOR signaling.

From the CFU-E stage onward, erythroblasts undergo a series of three to six terminal cell divisions and a morphological transformation. This stereotypical program is characterized by a decrease in cell size,⁶⁵ mitotic arrest,⁶⁶ nuclear condensation and expulsion, global changes in gene expression and mRNA splicing,^{67,68} and a dramatic increase in globin gene expression.⁶⁹ Caspase-3 and -7 are transiently activated^{1,70} and may have a role in erythroid maturation. In this regard, either siRNA knock-down of caspase-3 or caspase inhibitors cause a differentiation block at the proerythroblast stage, but caspase inhibitors have no effect at later stages of erythroid differentiation.^{70,71} Consistent with the latter, there is no

relationship between cleavage or degradation of nuclear subcompartment proteins and developmentally regulated nuclear condensation.⁷²

Nascent mammalian reticulocytes generated by the enucleation of mature erythroblasts reside in the bone marrow for 24 hours, are released into circulation, and complete differentiation over 2 to 3 days to form mature erythrocytes.^{73,74} During this time, reticulocytes change from large, motile, multilobular cells to small, biconcave, discoid cells⁷⁵; lose surface area and volume⁷⁶; undergo cytoskeletal and membrane remodeling⁷⁷; and eliminate ribosomes and membrane-bound organelles.^{78,79} Autophagy, or programmed self-digestion, is the process of envelopment of cytoplasm and organelles by double-membraned vesicles and delivery to lysosomes.⁸⁰ Ultrastructural studies suggest that during reticulocyte maturation, mitochondria are cleared by an autophagy-related process.^{78,81} Further, recent studies show that programmed mitochondrial clearance in reticulocytes depends on the autophagy protein, ULK1,⁸² and the proapoptotic BH3-only protein, NIX.^{83,84}

BCL-X_L and NIX are coordinately upregulated during terminal erythroid differentiation (Figure 30-2),^{83,85} and it is suggested that NIX may regulate erythrocyte production by providing a proapoptotic signal that opposes BCL-X_L.⁸⁶ However, contrary to this notion, the erythroid defect caused by BCL-X_L deficiency is not rescued by loss of NIX (unpublished results). Another possibility, consistent with the role of NIX in mitochondrial clearance, is that upregulation of NIX and BCL-X_L may simultaneously serve to promote cellular remodeling and to protect late erythroid cells from apoptosis. In this regard, there are at least three potential mechanisms through which NIX may function. First, in keeping with the well-established function of other BH3-only proteins, NIX may cause mitochondrial depolarization, which in turn causes mitochondrial autophagy.⁸⁴ However, if this is the mechanism, then it must occur independent of BAX, BAK, and the mitochondrial permeability transition pore, because none of these is required for mitochondrial clearance.⁸³ Second, NIX may function as an adaptor protein to recruit components of the autophagy or membrane trafficking machinery to mitochondria. Third, by analogy to other BH3-only proteins,⁸⁷ NIX may activate autophagy by competing with the autophagy protein Beclin-1 for binding to BCL-X_L. One of these mechanisms may be employed as erythroid cells switch from an apoptosis-prone to an autophagy-prone state during terminal differentiation.

Mature human erythrocytes in circulation have a lifespan of approximately 120 days.¹ Erythrocyte changes associated with senescence include membrane

loss, spherocytic shape change, and phosphatidylserine externalization.⁸⁸ By analogy to apoptotic cells, one idea is that externalization of phosphatidylserine may signal macrophages to engulf senescent erythrocytes. A similar mechanism is suggested for the DNase II-dependent clearance of expelled erythroblast nuclei by macrophages.⁸⁹ Erythrocytes contain caspase-3 and -8, but these are not activated even after prolonged storage; thus caspase activation does not appear to underlie erythrocyte senescence.⁹⁰ Rather, an increase in intracellular calcium and the activation of other cysteine proteases such as calpain is thought to cause the cellular changes that mark erythrocytes for clearance.

5. MEGAKARYOPOIESIS

Megakaryocytes, which produce platelets, are the other lineage that develops from MEP progenitors. Like the erythroid lineage, megakaryocyte development passes through early burst-forming progenitor and late colony-forming progenitor stages.⁹¹ Megakaryopoiesis is supported by the cytokine thrombopoietin, and mice deficient for the thrombopoietin receptor, c-Mpl, exhibit severe thrombocytopenia.⁹² Megakaryocytes represent less than 1% of the cells in the bone marrow. Because of a process known as endoreduplication, megakaryocytes have between a 4N and a 128N DNA content. Mature megakaryocytes have a high degree of polyploidy, and generate filamentous projections known as proplatelets. These platelet precursors project into the bone marrow sinusoids, and through a poorly understood process, release platelets into circulation. Platelets in circulation have a lifespan of about 10 days.

There are several steps of megakaryocyte development, which are regulated by death pathways. First, caspase activation is thought to have a role in proplatelet formation. Mature megakaryocytes, at the stage when proplatelets are formed, show caspase-9 and -3 activation.⁹³ Further, either enforced expression of BCL2 or caspase inhibition decreases, and FASL increases, proplatelet formation.^{94,94} Intriguingly, the process is compartmentalized; caspase activation is focal during proplatelet formation, but becomes generalized as denuded megakaryocytes undergo apoptosis.⁹⁵ Tg(*Vav-Bcl2*), *Bim*^{-/-}, and *Bax*^{-/-}; *Bak*^{-/-} mice have decreased platelet counts.^{34,35,36} implying a role for the intrinsic death pathway in platelet production, but these results are difficult to interpret because of potential effects on nonmegakaryocytic lineages. In this regard, it is notable that Tg(*Pf4-BclX_L*) mice, in which BCL-X_L transgene expression is restricted to the megakaryocyte lineage, show impaired platelet fragmentation.⁹⁶ Still, at present

it is unresolved whether caspase activation at the proplatelet stage is mediated through the intrinsic or extrinsic pathway.⁹⁴

Second, on activation, platelets exhibit some of the features of apoptosis, including cell shrinkage, plasma membrane microvesiculation, phosphatidylserine externalization, and proteolysis of procaspase-9, procaspase-3, gelsolin, and protein kinase C- δ .⁹⁷ However, in contrast to apoptosis, these events are not associated with cytochrome c release or caspase activation, but instead are mediated by the calcium-dependent cysteine proteinase, calpain. Third, platelet senescence is regulated through the intrinsic apoptotic pathway. BCL-X_L is upregulated during megakaryocyte maturation up to the stage of proplatelet formation; thereafter it is downregulated and distributed to developing platelets.^{96,98} BCL-X_L in circulating platelets is gradually degraded, placing a firm limit on platelet lifespan.⁹⁹ Once BCL-X_L levels fall below a critical threshold, BAK is activated, and cytochrome c released. Whether the final step leading to clearance is caspase-dependent or not is unclear.^{94,97}

6. GRANULOPOIESIS

Granulocytes and monocytes diverge from the erythroid and megakaryocytic lineages at the common myeloid progenitor (CMP); the CMP gives rise to bipotential colony-forming unit granulocyte-macrophage (CFU-GM) progenitors, which in turn give rise to the lineage-restricted progenitors CFU-G and CFU-M. Studies of genetically modified mice show that cytokines have multiple roles in the regulation of granulopoiesis (Figure 30-3). *Gcsfr*^{-/-} mice have decreased CFU-Mix, CFU-GM, and BFU-E, indicating an effect of granulocyte colony-stimulating factor (G-CSF) receptor signaling on commitment to the myeloid-erythroid lineage or the survival of myeloid-erythroid progenitor cells.¹⁰⁰ Additionally, *Gcsfr*^{-/-} mice have decreased mature neutrophils in the bone marrow and increased apoptotic neutrophil precursors, indicating an effect of cytokine signaling on neutrophil precursor cell survival.¹⁰¹ Consistent with a role for the intrinsic death pathway in these effects, myeloid progenitors or precursors are markedly decreased in *Mcl1* deleted mice²⁶ and moderately increased in Tg(*Vav-Bcl2*) mice,³⁴ Tg(*Mcl1*) mice,¹⁰² *Bim*^{-/-} mice,³⁵ and *Bax*^{-/-}; *Bak*^{-/-} mice.³⁶ Additional insight comes from mice that possess a conditional mutation of *Mcl1* and a monocyte-granulocyte lineage-restricted *LysM-Cre* transgene. *Mcl1*^{*fl/fl*};Tg(*LysM-Cre*) mice are neutropenic, showing that in addition to its requirement at the stem and progenitor cell stages, MCL1 is required for mature neutrophil

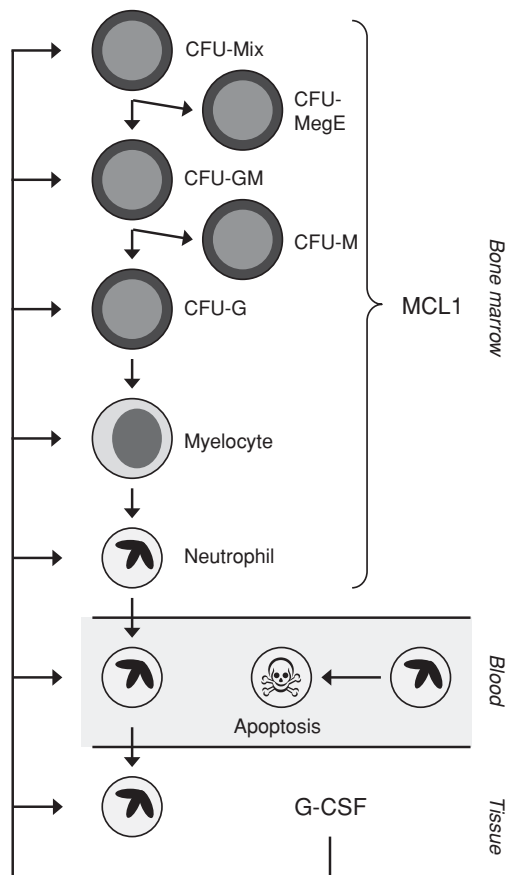


Figure 30-3. Regulation of granulopoiesis by G-CSF. G-CSF regulates granulopoiesis at multiple steps: progenitor and precursor cell survival in bone marrow, release from the bone marrow into the circulation, and neutrophil survival in tissues. Unstimulated neutrophils in circulation undergo constitutive apoptosis. MCL1 is required for progenitor survival and also specifically for terminal granulocytic differentiation. CFU-GM, bipotential granulocyte-macrophage progenitor; CFU-MegE, bipotential erythroid-megakaryocytic progenitor; CFU-Mix, multipotential progenitor; G-CSF, granulocyte-colony stimulating factor; Myelocyte, neutrophil precursor.

development.^{103,104} Finally, cytokine levels are markedly elevated in *Mcl1^{fl/fl};Tg(LysM-Cre)* and *Gcsfr^{-/-}* mice,¹⁰⁰ consistent with feedback regulation of neutrophil production.

In addition to promoting neutrophil precursor cell survival, cytokines also regulate the release of neutrophils from the bone marrow. Infection causes a marked increase in the systemic levels of G-CSF, macrophage colony-stimulating factor (M-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF).¹⁰⁵ Additionally, infection or pharmacological G-CSF causes the release of neutrophils and neutrophil precursors from the bone marrow into circulation and neutrophilia.^{106,107,108,109} Still, although cytokines have these properties, *Gcsf^{-/-}*, *Gcsfr^{-/-}*, *Gmcsf^{-/-}*, *Mcsf^{-/-}* and *Gmcsf^{-/-};Mcsf^{-/-}* mice generate neutrophils, indicating

a nonessential role, or redundancy, of these factors in neutrophil development.^{110,111,112,113,114}

Neutrophils undergo constitutive apoptosis and have a short lifespan in circulation (Figure 30-3).^{115,116} In vitro, in the absence of cytokines or factors, most neutrophils undergo apoptosis within 24 hours. Akt signaling is important for neutrophil survival. In the absence of cytokine signaling, dissociation of heat shock protein 27 and MAPK-activated protein kinase-2 from Akt-containing complexes prevents Akt activation and causes neutrophil commitment to apoptosis.^{117,118} Several lines of evidence suggest that the intrinsic death pathway also has a role in constitutive apoptosis. First, proapoptotic BCL2-related proteins are expressed in mature neutrophils, including BAX, BAK, BIK, BAD, and BID,¹¹⁹ but the antiapoptotic proteins BCL2 and BCL-X_L are not expressed, and MCL1 and A1 are expressed, but downregulated. Additionally, MCL1 protein is downregulated through proteasomal degradation.¹²⁰ Second, targeted disruption of *A1a*, one of three functioning and highly related *A1* genes in mice,¹²¹ hastens the onset of constitutive neutrophil apoptosis, and enforced expression of BCL2, or targeted disruption of *Bim*, retards it.^{122,123,124} Third, mitochondrial depolarization precedes phosphatidylserine exposure and other signs of neutrophil apoptosis.¹²⁵ Fourth, although the ability of broad-spectrum caspase inhibitors to prevent constitutive neutrophil apoptosis is controversial, caspase-3 is cleaved and activated during neutrophil apoptosis.^{126,127,128} Finally, despite expression of FAS by neutrophils^{129,130} and evidence for a mitochondria-independent mechanism,¹²⁷ constitutive neutrophil apoptosis does not appear to be regulated by FAS.^{123,128,131}

Circulating neutrophils are recruited to sites of infection by chemotactic factors and cross the endothelium by diapedesis.¹³² In tissues, neutrophils are exposed to local cytokines and inflammatory mediators, such as G-CSF, GM-CSF, lipopolysaccharide (LPS), C5a, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), adenosine triphosphate, leukotriene B₄, interleukin (IL)-1 β , IL-2, IL-3, IL-6, IL-15, and interferon- γ .¹³³ These factors activate survival and death pathways, which both prolong neutrophil survival and limit the duration of the immune response. For example, as noted above, Akt activity declines in unstimulated neutrophils leading to apoptosis¹¹⁷; however, G-CSF, GM-CSF, interferon- γ , and leukotriene B₄ activate Akt and inhibit apoptosis.^{117,134} Also, bacterial products and mimetics, such as LPS, peptidoglycan, and unmethylated CpG-DNA, inhibit apoptosis by activating Toll-like receptors, nuclear factor-kappa B (NF- κ B), and

Akt.¹³⁵ Finally, G-CSF, GM-CSF, IL-1 β , and LPS signaling increase levels of MCL1, A1, and phosphorylated BAD, suggesting that one or more of these are targets of their antiapoptotic activity.^{135,136} Other factors such as hypoxia and endothelial cell transmigration may also delay apoptosis and contribute to prolongation of neutrophil lifespan in tissues.^{137,138} In contrast, the death receptor ligands FASL and TNF- α , secreted by nearby macrophages, induce neutrophil apoptosis, which limits the duration of the immune response.^{131,139} In this regard, it should be noted that TNF- α can also inhibit apoptosis by activating NF- κ B.^{140,141}

Whereas the net effect of cytokines and inflammatory mediators in tissues is to prolong neutrophil survival, once neutrophils encounter bacteria, a series of events is initiated that ultimately leads to neutrophil clearance.¹⁴² When neutrophils encounter bacteria in tissues, the bacteria are phagocytosed and degraded. This is accomplished by the formation of phagosomes around bacteria and fusion with neutrophil granules and lysosomes to form a phagolysosomes. Within phagolysosomes, hydrogen peroxide, generated by the membrane-bound NADPH oxidase complex, is converted to hypochlorous acid and ROS.^{143,144} Neutrophils rely on glycolysis for energy,¹⁴⁵ but oxygen is consumed to generate ROS; consequently, this event is known as the respiratory burst. The importance of the respiratory burst for bactericidal activity is illustrated by the susceptibility of patients with NADPH oxidase mutations to infection, a condition known as chronic granulomatous disease.^{146,147} Once neutrophils ingest and kill bacteria, they are removed by macrophages. This serves the dual functions of eliminating bacteria and limiting the immune response.

Once bacteria are ingested, it is suggested that ROS, generated during the respiratory burst, may trigger neutrophil apoptosis. In support of this view, ingestion of heat-inactivated *Escherichia coli* induces neutrophil apoptosis, which can be inhibited by antioxidants.¹⁴² In addition, NADPH oxidase-mutant neutrophils, which cannot generate a respiratory burst or ROS, exhibit diminished neutrophil apoptosis¹⁴⁸ and accumulate in the tissues of patients with chronic granulomatous disease. On the other hand, ROS may target proapoptotic proteins for degradation, such as caspases, and therefore have an antiapoptotic effect,¹²⁸ and bacterial ingestion can actually inhibit neutrophil apoptosis.¹⁴⁹ Interpretation of these studies is complicated by the exceedingly short lifespan of unstimulated neutrophils in vitro; indeed, the demise of activated neutrophils in vivo is mediated at least in part by oxidant-induced phosphatidylserine exposure and pha-

gocytosis by macrophages, not caspase-dependent apoptosis per se.^{128,150}

7. MONOPOIESIS

Monocytes, which differentiate into tissue macrophages, are an important component of the innate immune response. Like granulopoiesis, monopoiesis is regulated by the effect of cytokines, including GM-CSF and M-CSF, on monocyte progenitor survival. Also, similar to the effect of G-CSF deficiency on neutrophils, M-CSF deficiency is associated with a significant decrease in macrophages.¹¹³ Still, *Gmcsf*^{-/-}, *Mcsf*^{-/-}, and *Gmcsf*^{-/-};*Mcsf*^{-/-} mice generate macrophages, indicating a nonessential role, or redundancy, of these factors in monocyte development.^{112,113,114}

Similar to neutrophils, monocytes exhibit a high rate of constitutive apoptosis and spend a short time in circulation, about 32 hours^{151,152}; however, once monocytes differentiate into macrophages, they are long-lived (days to weeks) and relatively resistant to apoptosis.¹⁵² Compared with neutrophils, monocytes are relatively resistant to FAS-induced death. This is attributed to the presence of BCL2¹²⁹ and an inhibitor of FAS signaling, FADD-like interleukin-1 beta-converting enzyme-inhibitory protein, which is upregulated during monocyte differentiation.¹⁵² Also, Akt is constitutively active in macrophages and associated with upregulation of MCL1.¹⁵³

The role of macrophages in the innate immune response is to phagocytose and kill bacteria and other cells, present antigen to lymphocytes, secrete cytokines that recruit neutrophils and prolong their survival, and secrete death receptor ligands that limit the duration of the immune response. To facilitate the destruction of phagocytosed bacteria, especially facultative intracellular pathogens, monocytes and macrophages undergo caspase-dependent apoptosis.^{149,154} Macrophages that have ingested bacteria undergo apoptosis, after an initial delay, which is attributed to a transient rise in MCL1, followed by expression of a proapoptotic dominant-negative MCL1 isoform.¹⁵⁵ Macrophage apoptosis after ingestion of bacteria is also attributed to Toll-like receptor-dependent upregulation of the proapoptotic BH3-only protein BIM¹⁵⁶ and to FASL secretion.¹³¹ Finally, it is suggested that ingestion of bacteria by macrophages may cause cathepsin D-dependent activation of caspase-3 and -7 and mitochondria-independent apoptosis.¹⁵⁷ Apart from its bactericidal role, macrophage apoptosis and uptake by antigen-presenting dendritic cells also serves to stimulate the adaptive immune response.^{158,159}

Given the importance of macrophage apoptosis in the innate immune response, it is not surprising that pathogens have evolved mechanisms to evade and manipulate this host defense. For example, some bacteria secrete pore-forming exotoxins that cause early macrophage death, others induce apoptosis after ingestion through inhibition of NF- κ B or MAPK signaling, and still others induce a form of programmed cell death, called pyroptosis, through caspase-1 activation.¹⁶⁰ By inducing premature apoptosis, bacteria diminish the killing activity of macrophages. Another, and opposite, strategy employed by facultative intracellular pathogens, such as *Mycobacterium tuberculosis*, is to inhibit macrophage apoptosis. *M. tuberculosis* infection of macrophages is associated with upregulation of MCL1 and resistance to apoptosis,¹⁶¹ which, combined with its ability to interfere with phagosome-lysosome fusion,¹⁶² permits its growth and spread inside host cells.

There is also an emerging appreciation of the role of autophagy in the innate immune response. Intracellular viruses, bacteria, and protozoa are all targeted by the autophagy machinery.¹⁶³ Autophagy is especially important in the defense against facultative intracellular pathogens. Stimulation of autophagy improves *M. tuberculosis* clearance by directing mycobacterial phagosomes to the autophagy pathway for degradation.¹⁶⁴ Even without formation of typical double-membraned autophagosomes, phagocytosis of bacteria and Toll-like receptor signaling promotes recruitment of autophagy proteins to phagosomes, phagosome fusion with lysosomes, and phagosome maturation.^{165,166} As is the case for apoptosis, pathogens have evolved adaptations to subvert autophagy-dependent defenses.¹⁶³

8. CONCLUSION

An overview has been provided of the physiologic roles of cell death pathways in hematopoiesis. The studies covered here illustrate several points about these pathways. First, proper regulation of apoptosis is essential for homeostasis in the hematopoietic system and presumably other tissue types as well. Second, although the effect of death pathways is often represented as a simple scale with only two possible outcomes (death or survival) these pathways are used in vivo in entirely different ways to achieve specific outcomes. Thus cell division and apoptosis are repressed to prevent depletion of HSCs, apoptosis regulates the exponential expansion of hematopoietic progenitors under normal and stress conditions, and apoptosis in neutrophils and macrophages is bactericidal. These pathways are highly regulated and respond to extracellular signals from the

local environment and distant sites. Third, although it remains somewhat speculative, death pathways appear to be involved in the regulation of other cell fates, such as autophagy. One important line of investigation in the future will be to understand how the differentiation program controls the transition between different states; for example, from a state where apoptosis is repressed to one where it is favored, or from a state where apoptosis is favored to one where autophagy is favored. Elucidation of the mechanisms underlying these state changes is important for our understanding of cellular differentiation, and the hematopoietic system is an ideal model in which to work.

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1. INTRODUCTION

More than 210,000 people die from sepsis in the United States each year, with an annual cost of more than 16 billion dollars.^{1,2,3} Despite continued advances in treatment and prevention, sepsis is a growing problem, with a significant mortality rate of 28% to 50%.^{2,4} In the past, death from sepsis was thought to be due to uncontrolled inflammation, and as a result, numerous anti-inflammatory therapeutics were developed. Uncontrolled inflammation leading to death may be true in sepsis due to certain types of pathogens (e.g., *Neisseria meningitidis*, *Clostridium perfringens*)⁵ and in these patients anti-inflammatory therapies may help. However, large-scale clinical trials of anti-inflammatory therapies in septic patients have failed to reduce patient mortality.¹ Recent research into the host's immune response in sepsis has led to a fundamental change in the way clinicians and researchers think about this disease.⁶ After an initial hyper-inflammatory phase, septic patients may descend into a period of prolonged immune suppression, and it is during this period that the majority of patients die.⁷ Death usually occurs from multiorgan system failure brought on by the host's inability to clear the primary infection or from a second opportunistic or nosocomial infection. One important hallmark of sepsis is widespread cell death in multiple organ systems due to both apoptosis and necrosis.⁶ This chapter reviews the cell types that undergo apoptosis and necrosis, the known inciting factors and mechanisms of cell death, and the impact of sepsis-induced apoptosis on morbidity and mortality, especially focusing on the importance of the lymphocyte.

2. HOST INFLAMMATORY RESPONSE TO SEPSIS

Sepsis causes canonical inflammation in the immune competent host. Celsus, a Roman writer of the first century CE, was the first to enumerate the four cardinal signs of inflammation: *rubor*, *tumor*, *calor*, and *dolor* (redness, swelling, heat, and pain). A fifth clinical sign, loss of function (*functio laesa*), was later added by Virchow. With the advent of cellular and molecular biology, we now have an etiologic basis of these clinical findings.

Researchers have shown in both animal models of sepsis and in septic patients that there is a complex, multiphasic inflammatory program.⁸ There are two competing models: one camp has proposed that sepsis is a constant mixed inflammatory state that has different local and systemic effects, whereas the other view is that sepsis has discrete hyper- and hypo-inflammatory phases.^{6,9} The initial phase is predominantly hyper-inflammatory and is characterized by fever, hypermetabolism, muscle protein breakdown, and decreased vascular resistance.⁸ This phase is driven by proinflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-6, which are released by cells of the innate immune system, including activated macrophages.⁸ As sepsis progresses, patients descend into a hypo-immune phase, which is dependent on many factors, including the virulence of the pathogen, the amount of bacterial inoculum, genetic background (polymorphisms), host comorbidities, and so forth.⁶ In this phase of sepsis, both the innate and adaptive immune system are compromised, evidenced by loss of delayed-type hypersensitivity response (anergy),¹⁰ a shift from a Th1 phenotype to a Th2 phenotype,^{11,12} increase in the proportion of T-regulatory cells,^{13,14} decreased major

Table 31-1. Mechanisms of immune dysfunction

Apoptosis of T cells, B cells, dendritic cells and monocytes
TH1 → TH2 phenotype
Anergy
Increased proportion of T-regulatory cells
Increased anti-inflammatory mediators
Loss of macrophage expression of MHC-II and costimulatory molecules
Deactivated monocytes

MHC, major histocompatibility complex.

histocompatibility complex II expression,^{15,16,17} increase in anti-inflammatory mediators,¹⁸ decreased monocyte expression of human leukocyte antigen type DR,⁷ and the apoptotic loss of lymphocytes, dendritic cells, and gastrointestinal epithelial cells⁶ (Table 31-1). Immunosuppressed patients fail to clear their primary infections and are susceptible to secondary infections, either nosocomial or opportunistic; survival is correlated with the ability to maintain or restore immune competence.⁷

3. CLINICAL OBSERVATIONS OF CELL DEATH IN SEPSIS

3.1. Sepsis-induced apoptosis

Sepsis-induced apoptotic cell death was first characterized in a study in which autopsies were conducted in critically ill patients who died of either sepsis or nonseptic-related etiologies.¹⁹ Autopsies were performed in the intensive care units 30 to 90 minutes postmortem to avoid cell autolysis after death. The causes of sepsis were multifactorial, with a predominance of patients having nosocomial pneumonia. Most patients suffered from multiorgan system failure and experienced extended periods of hypotension requiring vasopressor treatment. Patients who died from sepsis had extensive apoptotic death of splenic lymphocytes and gastrointestinal epithelial cells compared with critically ill patients who died from nonseptic causes (Figures 31-1 and 31-2). Other organs, including lung, kidney, and skeletal muscle, did not reveal consistent apoptosis or necrosis despite a majority of patients exhibiting multiorgan dysfunction.

Apoptosis was detected by three different techniques that identify differ-

ent features of apoptotic cells: (1) histologic evaluation of hematoxylin/eosin-stained tissue sections revealed characteristic pyknotic nuclei and karyorrhexis, (2) fluorescent terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining demonstrated systematic genomic degradation subsequent to poly (ADP-ribose) polymerase activation, and (3) immunohistochemical staining identified the active form of the executioner caspase, caspase-3. Light microscopy further revealed massive loss of lymphocytes and disruption of germinal center architecture from the spleens of septic patients compared with nonseptic controls. Subsequent studies revealed that the cells that were depleted during sepsis were CD4⁺ T cells, B cells, and follicular and interdigitating dendritic cells.^{20,21} Septic patients were also found to have decreased circulating lymphocyte counts compared with nonseptic patients. Immune cell apoptosis in septic patients has been demonstrated in subsequent autopsy studies in both children and neonates.^{22,23}

Septic patients have a marked increase in apoptosis of circulating lymphocytes when compared with critically ill nonseptic patients.²⁴ Apoptosis leads to lymphopenia that is persistent in septic patients, and the degree of apoptosis is positively correlated with the severity of sepsis and with poor outcome. Recent studies looking at immune cells from septic patients found significant upregulation of the messenger RNA for the

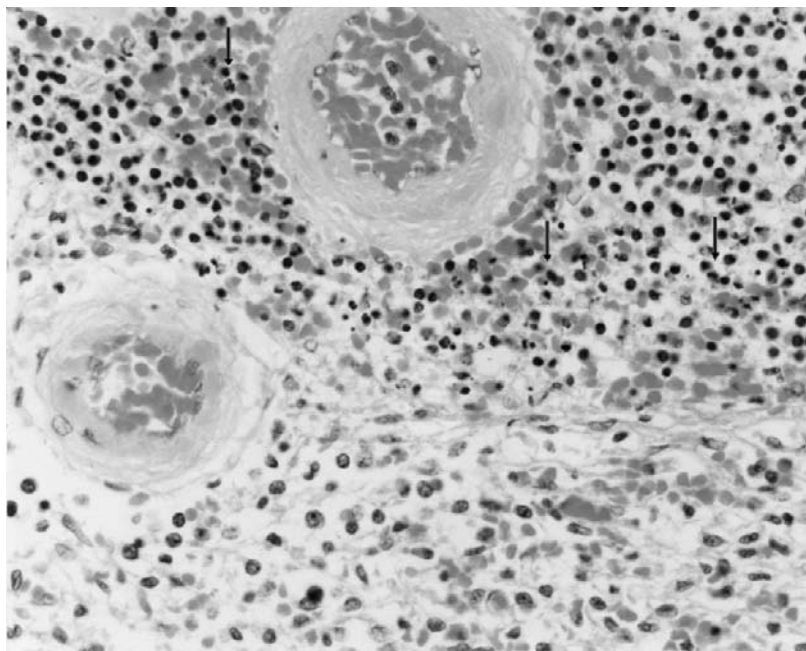


Figure 31-1. Lymphocyte apoptosis in spleen of septic patient. Hematoxylin and eosin staining of spleen of septic patient (400× magnification). Note the abnormal, pyknotic nuclei and nuclear debris characteristic of apoptotic cells (arrows). See Color Plate 35.

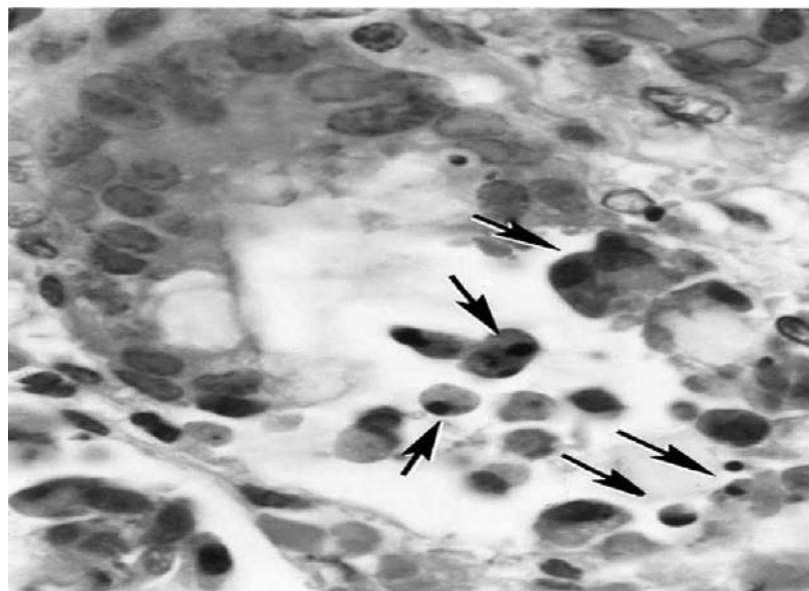


Figure 31-2. Colonic epithelial apoptosis in a septic patient. Hematoxylin and eosin staining of colonic epithelium of a septic patient (400 $\times$ magnification). Arrows point to apoptotic cells being shed into the lumen of the colon. See Color Plate 36.

proapoptotic genes Bid, Bim, and Bak and downregulation of BCL-2.²⁵

In addition to the hematopoietic compartment, the gastrointestinal epithelium has long been a focus of study in sepsis research, and the gut has even been referred to as the “motor” of the immune system.²⁶ The lining of the gastrointestinal tract “turns over” every 3 to 5 days and is a function of the balance between cell death and proliferation. Maintenance of this barrier is important in preventing translocation of live bacteria or bacterial toxins. The aforementioned autopsy study identified the increased incidence of colonic epithelial apoptosis in both the villi and crypts in septic patients compared with nonseptic patients (Figure 31-2).¹⁹ Apoptosis of epithelial cells was also seen in the ileum of septic patients. Gastrointestinal epithelial apoptosis may lead to breakdown of this important barrier, resulting in systemic leakage of endogenous flora.

3.2. Necrotic cell death in sepsis

These autopsy studies also revealed necrotic cell death in other organ systems in patients who died with sepsis.¹⁹ Microscopic evidence of necrosis in the liver was identified in approximately 33% of patients. Interestingly, apoptotic hepatocytes were seen in some patients with identified necrosis, and the apoptosis occurred in proximity to necrotic foci. It is important to note that almost all of the patients with sepsis were in septic shock

and were requiring vasopressors to maintain an adequate arterial blood pressure. The area of the liver that demonstrated necrotic changes was the region approximating the central vein. This region is vulnerable to hypoxia and decreased flow because of its unique perfusion. Thus the hepatocyte necrosis may have been secondary to hypotension and hypoxia in the setting of sepsis. Necrotic cell death was also seen in the brain and heart of three patients. However, these patients had evidence of prior cerebrovascular incidents or myocardial infarction.

Although these studies did not conclusively link sepsis to necrotic cell death, there was unquestionable necrosis within hypoxia-sensitive tissues. This necrosis could be explained by ischemia-reperfusion injury, as there is well-documented microvascular patho-

logy in sepsis; however, the role of necrosis in sepsis remains poorly understood.

4. THE DEVELOPMENT OF CLINICALLY RELEVANT ANIMAL MODELS OF SEPSIS

Early animal models of sepsis, on which previous research and therapies were based, involved lipopolysaccharide challenge (intravenous, intratracheal, or intraperitoneal).²⁷ These models defined the classic proinflammatory phase of sepsis, characterized by tachycardia, tachypnea, hypotension, and high levels systemic of TNF- α , IL-1, IL-6, and interferon (IFN)- γ , and anti-inflammatory interventions in these models resulted in significant gains in survival. Unfortunately, anti-inflammatory strategies based on these models failed to provide significant mortality benefit to the general septic population.¹ Failure of these therapies led to development of more clinically relevant models of sepsis. The cecal-ligation and puncture (CLP) model was developed to mimic sepsis owing to a ruptured appendix or bowel perforation.²⁸ Pneumonia models use bacteria commonly found to cause pneumonia, such as *Pseudomonas aeruginosa* or *Streptococcus pneumoniae*.²⁹ Pneumonia after CLP – or “two-hit” – models of sepsis mimic clinical scenarios involving secondary, or nosocomial, infections.³⁰

The identification of immune cell and gastrointestinal epithelial apoptosis in septic patients led researchers

to investigate whether clinically relevant models of sepsis also exhibited these findings. Animal models have provided important insights into the role of apoptosis in the pathophysiology of and mortality from sepsis.

4.1. Central role of apoptosis in sepsis mortality: immune effector cells and gut epithelium

The balance between pro- and antiapoptotic proteins, especially Bcl-2 and its family members, regulates apoptotic cell death.^{31,32,33} Experiments using transgenic mice have provided mechanistic insights into the role of apoptosis in sepsis lethality. Mice over-expressing Bcl-2 in T and B lymphocytes are resistant to sepsis-induced lymphocyte apoptosis and have improved survival after cecal ligation and puncture when compared with wild-type mice.^{34,35} Mice over-expressing Bcl-2 in the gastrointestinal epithelium have decreased gut epithelial apoptosis and improved survival in a model of *Pseudomonas* pneumonia.³⁶ Sepsis probably creates an environment that accelerates death of gut epithelial cells that are predestined to die and initiates the apoptotic machinery in other cells. During pneumonia there is a disassociation between apoptotic cell death and cellular regeneration and a large number of intestinal epithelial cells undergo apoptosis, but there is not a compensatory increase in gut epithelial cell proliferation.³⁷ These studies, along with research delineating apoptotic pathways (vide infra), highlight the significant role of apoptotic cell death in sepsis.

4.2. Apoptotic pathways in sepsis-induced immune cell death

Apoptosis in mammalian cells is mediated through two different pathways.³¹ The extrinsic, or death receptor, pathway is activated by a number of death receptor ligands, including TNF- α , TNF-related apoptosis-inducing ligand (TRAIL), and Fas ligand and act through the death-inducing signaling complex (DISC), which activates the initiator caspase, caspase-8. The intrinsic, or mitochondrial-mediated, pathway can be activated by a large number of stimuli, including oxidative stress, radiation, cytochrome c, cytokine withdrawal, and chemotherapeutic agents. Activation of this pathway results in formation of the apoptosome (a macromolecular assembly of apoptotic protease activating factor 1, cytochrome c, and pro-caspase-9), which activates the initiator caspase, caspase-9. Once activated, caspase-8 and caspase-9 can cleave the executioner caspase, caspase-3, which in turn activates a cascade of

proteases and endonucleases that results in the systematic disassembly of the cell. Current evidence suggests that both pathways are involved in sepsis-induced lymphocyte apoptosis.³⁸

4.3. Investigations implicating the extrinsic apoptotic pathway in sepsis

A key protein in the assembly of the DISC is the Fas-associated death domain (FADD). Mice that express a dominant-negative form of FADD in T cells are protected from T- and B-cell apoptosis and have increased survival compared with wild-type mice in the CLP model of sepsis.³⁹ Mice deficient in Fas ligand have decreased B-cell apoptosis during sepsis. Inhibition of Fas/FasL signaling has been shown to increase survival in sepsis and prevented loss of macrophages.⁴⁰ Apoptosis in CD4 T-cell populations can also be mediated by FasL during polymicrobial sepsis.⁴¹ These results point to the multiplicity of death stimuli that are likely involved in sepsis-induced apoptosis via the extrinsic pathway. FADD integrates a large number of these signals, and therefore, removing its function is broadly protective; although removing a single ligand or receptor that signals through FADD can attenuate apoptosis, no single ligand/receptor pair studied phenocopies the survival advantage in FADD-DN mice. Interestingly, deletion of MyD88, another significant integrating node in the immune system that transduces pathogen sensing by Toll-like receptors, leads to amelioration of T- and B-cell apoptosis owing to sepsis but worsened survival in a CLP model of sepsis.⁴² These results indicate that certain classes of signal are essential for engaging the immune system, whereas other classes of signal molecules are detrimental. Mice deficient for MyD88 had essentially no cytokine production in sepsis and were therefore unable to respond to their infection.

4.4. Investigations implicating the intrinsic apoptotic pathway in sepsis

The mitochondrial pathway is activated by multiple stimuli and is mediated by the Bcl-2 family of proteins.³³ The Bcl-2 family of proteins includes more than 15 members and comprises both antiapoptotic and proapoptotic members. Bcl-2, which was characterized first, is known to function primarily as an inhibitor of apoptosis. Studies in both cancer and sepsis have used Bcl-2 to modulate apoptosis.³³ The importance of this protection in sepsis was first demonstrated by our laboratory in studies showing that transgenic over-expression of Bcl-2 in lymphocytes decreased immune effector cell apoptosis

Table 31-2. Impact of apoptosis on immune function

Decreased presentation of antigen to T cells
Decreased macrophage activation and depressed phagocytic capability
Impaired adaptive immune response
Disrupted cross-talk between adaptive and innate immune systems
Release of anti-inflammatory cytokines
T-cell anergy

and increased survival in murine models of sepsis,^{34,35} a finding that has been recapitulated by others.⁴³

The antiapoptotic Bcl-2 family members, of which Bcl-2 is a member, contain four Bcl-2 homology (BH) domains with sequence and structural homology. The hallmark of this protein family is the BH4 domain, a conserved N-terminal helical domain that is required for these proteins to perform their antiapoptotic functions.³³ The remaining Bcl-2 family members have proapoptotic activity. These proteins comprise two subfamilies classified by their BH domain composition. A handful of proteins contain BH1-BH3 domains, and the remainder (the largest Bcl-2 subfamily) contains only the BH3 domain. The BH1-BH3 containing family members have been shown to oligomerize and permeabilize the mitochondrial outer membrane, which leads to cytochrome c release and ultimately apoptosis.³³ Under basal conditions, these proteins are kept in an inactive reservoir, primarily through interactions with BH4 containing Bcl-2 family members. Although it is accepted that the BH3-only proteins trigger the activation of Bax and Bak, there is currently a controversy regarding the precise mechanism BH3 proteins employ to activate Bax/Bak.⁴⁴ Regardless of the mechanism, supporters of both models have demonstrated that Bim, Bid, and p53-upregulated modulator of apoptosis (PUMA) are the most potent BH-3 only proteins.³³

In CLP, knockout of Bim prevented apoptotic loss of lymphocytes in sepsis and improved survival, whereas knockout of PUMA provided modest protection against apoptosis but no survival advantage.³⁹ Bid is unique among the BH3-only proteins in that it mediates cross-talk between the extrinsic and intrinsic pathways. Caspase-8 cleaves Bid into truncated (t)Bid, which reinforces death receptor signaling by activating the intrinsic apoptotic pathway. Knockout of Bid in mice rendered lymphocytes resistant to sepsis-induced apoptosis and increased survival.⁴⁵

Examination of the apoptotic pathways in sepsis reveals that there is no single mediator or pathway that is responsible for lymphocyte apoptosis. Indeed, these

data reveal that lymphocytes have extensive apoptotic machinery and that sepsis creates an environment rich in death stimuli that can activate these processes. The type of cell, its activation state and phase of the cell cycle, and type of microorganism all appear to influence whether a lymphocyte will undergo apoptosis. On the basis of findings in animal models of sepsis, the BCL-2 family plays a central role in sepsis-induced apoptosis.

5. THE EFFECT OF APOPTOSIS ON THE IMMUNE SYSTEM

Lymphocyte apoptosis may play a role in the evolution of the inflammatory response that is seen in septic patients by modulating cellular function directly and also by disrupting the network of immune cell interactions that are necessary to mount an effective immune response (Table 31-2).

5.1. Cellular effects of an increased apoptotic burdens

Apoptotic cells themselves are immunosuppressive. Macrophages and dendritic cells that take up and eliminate apoptotic cells release anti-inflammatory (Th2-inducing) cytokines such as IL-10 and transforming growth factor beta (TGF- β) and suppress proinflammatory cytokines.⁴⁶ T cells that come into contact with these macrophages and dendritic cells become anergized or undergo apoptosis themselves. A systemic burden of apoptotic cells has been found to worsen survival in septic mice. Mice receiving adoptive transfer of apoptotic lymphocytes before CLP have worse survival in sepsis, whereas transfer of necrotic cells improves survival.⁴⁷ This differential may be due in part to how macrophages clear cellular debris and the resulting effect on IFN- γ production; phagocytic uptake of apoptotic cells by macrophages leads to a decrease IFN- γ production, whereas uptake of necrotic cells increases it.

5.2. Network effects of selective loss of immune cell types

Apoptosis of dendritic cells cripples the innate immune system by reducing the capacity to process and present antigen to the adaptive system. Loss of T and B cells disrupts the adaptive immune response and impairs the communication between the adaptive and innate arms of the immune system. The role of the adaptive immune system in sepsis has been described in studies using mice that lack T and B cells (Rag 1^{-/-} mice). Adoptive transfer of lymphocytes over-expressing Bcl-2 to Rag^{-/-} mice attenuates mortality owing to

sepsis.⁴⁸ Survival of immune cells in apoptosis improves survival in sepsis and may also promote the survival of other types of cells, such as neutrophils.⁴³ Adoptive transfer of myeloid (CD11b⁺) cells from transgenic mice over-expressing Bcl-2 in myeloid cells was found to improve survival, decrease gut epithelial apoptosis, and increase peritoneal neutrophil counts in Rag 1^{-/-} mice when compared with Rag 1^{-/-} receiving cells from wild-type C57/BL6 mice. These data suggest that survival of lymphocytes and myeloid cells may exert a “bystander” effect by releasing some sort of cytoprotective molecule that inhibits apoptosis of gut epithelium and other immune effector cells.

5.3. Studies of immunomodulation by apoptotic cells in other fields

The impact of apoptotic cells in sepsis is consistent with data from the transplant literature. The immunomodulatory effects of apoptotic cells were determined in a study using infusion of apoptotic cells in mice that later received bone marrow transplants.⁴⁹ The investigators found that apoptotic cell infusion caused a TGF- β dependent T-regulatory cell expansion, which in turn caused immunosuppressive effects via a cell contact mediated mechanism. In their model, the downregulation of the immune system had beneficial effects of decreasing graft-versus-host disease and increasing engraftment of donor bone marrow cells. Although beneficial in the case of bone marrow transplant, in an animal model of sepsis or in patients dying from sepsis, downregulation of the immune system could be catastrophic.

6. DEVELOPING THERAPIES TO AMELIORATE SEPSIS-INDUCED LYMPHOCYTE APOPTOSIS

As discussed previously, research over the last decade has established the important role that immune cell apoptosis plays in the response to sepsis. These findings have encouraged the development and application of several technologies to modulate apoptosis. Two different approaches to antiapoptotic therapies are under investigation – rational therapies using biologic agents (RNA, peptides, cytokines, and proteins) and screening-based discovery of novel cytoprotective agents. Rational therapies have been based on the knockout and transgenic mice that have well-documented survival advantages in animal models of sepsis.

Therapeutic recapitulation of the survival advantage observed in transgenic mice has required detailed molecular mechanistic understanding of apoptosis

pathways. For example, mice that over-express Bcl-2 or Bcl-xL in lymphocytes have a profound survival advantage in sepsis. Bcl-2 and Bcl-xL are cytoplasmic proteins that undergo BH4 domain-dependent trafficking from the endoplasmic reticulum to the mitochondrial surface with the help of FKBP38.⁵⁰ Unfortunately, these proteins and their BH4 effector domain are unable to cross cell membranes, making them unlikely therapeutic candidates.

However, intracellular delivery of membrane-impermeant materials was enabled by the discovery of cell permeation peptides including the Antennapedia homeodomain peptide and a short, polycationic peptide from the HIV protein Tat.⁵¹ Previous work revealed that Tat-mediated delivery of the BH4 domain of either Bcl-2 or Bcl-xL could protect cells from diverse apoptogenic stimuli, including chemotherapy,⁵² ischemia-reperfusion injury,^{53,54} and radiation injury.⁵⁵ Administration of antiapoptotic proteins such as BCL-2 and BCL-xL or their BH4 domains conjugated to the Tat peptide has been shown to be effective in preventing sepsis-induced lymphocyte apoptosis.⁵⁶

Therapies aimed at recapitulating the phenotypes of knockout mice require inhibiting the synthesis of proapoptotic proteins. Small interfering RNA is one exciting modality that has been shown to be effective in preventing lymphocyte apoptosis and improving survival in sepsis models. siRNA to Fas, caspase-8, and Bim have all been reported to decrease sepsis-induced apoptosis and mortality after CLP.^{57,58} Although the basic principles of RNA interference are well worked out, clinical applications will require additional technology. Most importantly, tissue-specific delivery agents that target the biodistribution and uptake of therapeutic nucleic acids must be developed to prevent undesirable side effects. At the writing of this chapter, two approaches appear to hold promise, biologic targeting via ligand (e.g., transferrin^{59,60}) or antibody (e.g., anti-CD4⁶¹) and materials-based targeting using different polymer compositions.^{62,63}

An alternative approach to rational biologic therapy is to develop pharmacological (small-molecule) modulators of essential apoptosis proteins. Previous work has shown that small-molecule caspase inhibitors can reduce sepsis-induced apoptosis⁴⁸; however, these molecules have not entered clinical study. One potential difficulty with caspase inhibition to prevent apoptosis is that caspases have recently been found to play a key role in the cell cycle.⁶⁴ Another potential challenge is that once executioner caspases are activated, cells have accumulated enough damage to no longer respond appropriately to their environment.

Cytoprotection can be accomplished in other ways besides caspase activation; however, with the exception of Bcl-2 family members and FADD, molecular mechanisms that contravene sepsis-induced lymphocyte apoptosis are not well-established. Instead of targeting a single enzyme or protein-protein interaction, high throughput screening of compound libraries using a cell phenotype assay can be employed to discover novel cytoprotective agents that can serve as lead compounds for drug development.

Immunotherapy is also emerging as a potential therapeutic strategy for the treatment of sepsis. Administration of cytokines, such as IL-7 or IL-15, or antibodies to negative co-stimulatory molecules (Programmed Death receptor-1) have been shown to prevent apoptosis, reverse immunosuppression, and improve survival in mice after CLP. Through modulation of BCL-2 and the prevention of lymphocyte apoptosis, these therapies may improve immune dysfunction seen in sepsis.^{65,66,67}

Although encouraging preclinical data have been developed for peptide, RNA, and small-molecule therapies, translating these findings from the laboratory to the clinic remains a significant challenge. It is likely that at least three key areas will need further research before clinical trials of antiapoptotic therapies. First, further development of lead compounds will need to be pursued. Second, we need a better understanding of how sepsis alters drug metabolism, pharmacokinetics, and pharmacodynamics. And finally, any antiapoptotic agent, even if administered a single time, has the theoretical risk of facilitating cellular transformation. Therefore, there will need to be a demonstration that short-term treatment with a candidate antiapoptotic therapy does not significantly increase the risk of cancer.

7. CONCLUSION

From premature infants to elderly patients requiring elective surgeries, the risk of sepsis continues to be a significant cause of morbidity and mortality around the world. Over the last decade, our understanding of the pathophysiology of sepsis has grown tremendously. Most patients dying from sepsis are now thought to die during a state of "immunoparalysis," not the initial inflammatory response. One of the key findings in the pathophysiology of sepsis has been the connection between immune effector cell apoptosis and prolonged immune suppression. Several research groups have reported dramatic improvements in survival in animal models of sepsis when apoptosis is prevented. These findings represent a significant impetus for pursuing clinical trials with antiapoptotic, immuno-

supportive agents to determine whether prevention of sepsis-induced apoptosis can change the course of disease in human patients. Hopefully, strategies to block sepsis-induced apoptosis will be able to decrease the morbidity and mortality from this highly lethal disorder.

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1. INTRODUCTION

In this chapter, I address the fundamental questions of what differentiates pathogens from commensal microorganisms and how pathogens succeed in causing infectious disease. I delve in more detail into host mechanisms evolved to detect the presence of invaders and to fight infections. I focus on the role of innate immunity, production of antimicrobial peptides, inflammation, and cell death in the host–pathogen battle and discuss the remarkable conservation of innate immunity between plants, invertebrates, and mammals, despite having evolved under selective pressure imposed by distinct pathogens.

2. FROM THE PATHOGEN PERSPECTIVE

2.1. Commensals versus pathogens

We live in a world laden with microbes. At birth, microorganisms populate our body, forming what is called the normal or microbial flora, which constitute 90% of the cells in our body. The composition of the flora is different among anatomical sites and varies among individuals depending on genetics, age, sex, diet, and stress. Microorganisms in our flora are called commensals. Sociologically, a *commensal* is defined as an “individual not competing while residing in or occupying the same area as another having independent or different values.” Literally, a commensal is a companion eating at the same table. Both definitions are indeed appropriate to describe our symbiotic relationship with commensal microorganisms. They share our nutrients and in return provide us with vitamins, aide in our digestion, and are an important component of our innate immune system,

where they compete with, and protect us from, invading pathogens.

Pathogenic and commensal bacteria share a common structure. They have a nucleoid, a cytoplasm, a plasma membrane, and a cell wall composed of proteins and carbohydrates. Some have additional features such as flagella that allow motility (Figure 32-1). Molecules that constitute the general structure of microbes are unique to them and are thus recognized by the host innate immune system as foreign or non-self. Because microbes evolve rapidly, innate immunity focuses on molecules, predominantly structural elements, which are common to broad classes of microbes. It is useful to think of them as patterns. These include, for example, lipopolysaccharide (LPS), also called endotoxin, found on the cell wall of Gram-negative bacteria; peptidoglycan, which forms approximately 90% of the dry weight of Gram-positive bacteria but only 10% of Gram-negative strains (Figure 32-2); nucleic acids; and flagellin, the main constituent of flagella. Although these patterns are common to both commensals and pathogens, they were named *pathogen-associated molecular patterns* (PAMPs). In theory, a host would equally recognize commensals and pathogens. However, our coexistence with commensals suggests otherwise and indicates the presence of intriguing host mechanisms that limit the recognition of commensals. PAMPs are sensed by germ-line encoded receptors in the host, called pattern-recognition receptors (PRRs). Downregulation or absence of PRR expression on the surface of epithelial cells that are in direct contact with commensals might be one such mechanism.

Commensals and pathogens differ in their ability to infect the host. Pathogens are capable of evading host defenses and breaching anatomical barriers such as

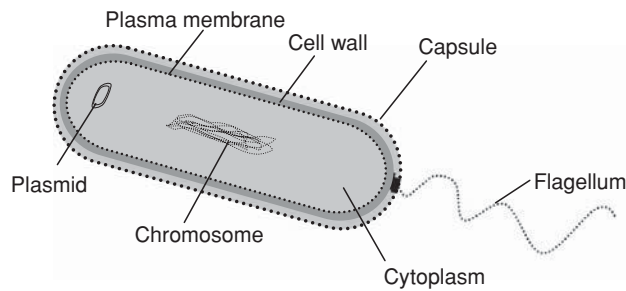


Figure 32-1. General structure of bacteria. Bacteria have a nucleoid, a cytoplasm, a plasma membrane, and a cell wall composed of proteins and carbohydrates. Some have additional features, such as a carbohydrate capsule or slime layer, which protects from phagocytosis, or flagella, which allow motility.

those of the skin or internal epithelial barriers. This permits them to reach the bloodstream and enter in otherwise sterile tissues such as the spleen or liver, from which commensals are excluded. This faculty is determined at the genomic level. Pathogens harbor genomic regions or “islands” of pathogenicity, consisting of gene clusters that encode virulence factors.¹ Collectively, these factors allow pathogens to go through their life cycles successfully; they function during invasion of the host, colonization, persistence, replication, and dissemination (Figure 32-3).

2.2. Pathogen strategies to infect the host

Pathogens have acquired sophisticated mechanisms to invade the host while evading phagocytosis and immune

surveillance. The first step after entry in the host is for pathogens to avoid physical expulsion and to colonize the site of infection. The best example to illustrate this is that of “attaching and effacing” bacteria, such as enterohemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* that enter the host through the oral route and colonize the colon by attaching to the surface of colonocytes and effacing their microvilli² (Figure 32-4a). Pathogens are then faced with the task of hiding from the immune system. Various strategies are used for this purpose. Extracellular pathogens remain in the host by evading phagocytosis. *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Neisseria meningitidis*, and uropathogenic *E. coli* surround their cell wall with a thick carbohydrate “capsule” that allows them to resist phagocytosis and complement-mediated lysis (Figure 32-4b). Many Gram-negative bacteria evolved modifications in their molecular patterns to avoid recognition by PRRs. Structurally, LPS is composed of a lipid A moiety that anchors polysaccharide O-antigen chains to the outer leaflet of the bacterial cell wall.³ In a complex with MD-2, the pattern recognition receptor TLR4 efficiently detects lipid A,⁴ which consists of hexa-acylated molecules with 12 to 16 carbon-fatty acid side chains.⁵ Various pathogens, including *Helicobacter pylori*,⁶ acquired changes in either the number of acyl groups or length of fatty acid side chains to interfere with recognition by TLR4. Similarly, a number of bacteria, notably mucosal pathogens, modulate the structure

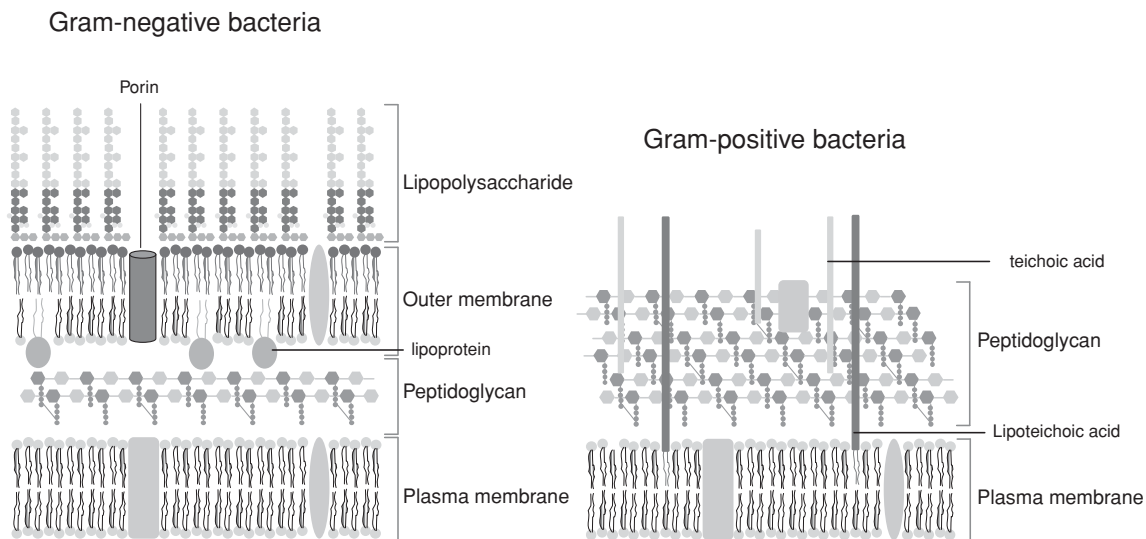


Figure 32-2. A representation of the cell wall from Gram-negative and Gram-positive bacteria. LPS is the main component of Gram-negative bacteria cell wall. Peptidoglycan constitutes 90% of the dry weight of Gram-positive bacteria but only 10% of Gram-negative strains. LPS, porin, lipoproteins, peptidoglycan, and teichoic acid are all recognized by the innate immune system through specific activation of PRRs. See Color Plate 37.

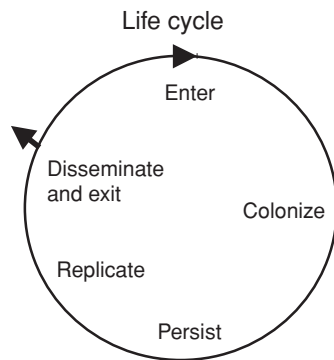


Figure 32-3. The life cycle of a pathogen. For a pathogen to survive it needs to invade the host, colonize the site of infection while resisting expulsion from the host, replicate, then exit to disseminate and find another host that would supply a new replication site and source of nutrients. Courtesy of Stanley Falkow.

or expression levels of flagellin when crossing epithelial cell barriers⁷; these cells express, on their basolateral surface, TLR5, a PRR that senses flagellin and guards against pathogen invasion.^{8,9}

For intracellular pathogens, the challenge is of a different nature. They need to enter host cells, replicate without being recognized and degraded, and then exit the cell. A common strategy of intracellular pathogens is subversion of the endocytic machinery to their advantage and hiding in endosomal compartments. For instance, *Brucella spp.*, as well as *Legionella pneumophila*, the causative agent of Legionnaire's disease, surround their phagosomes with an endoplasmic reticulum-derived membrane that inhibits phagosome-lysosome fusion, establishing an ideal niche for replication (Figure 32-4c).¹⁰ After replication, pathogens break out of their hiding places into the cytosol, where they induce cell death to exit the cell and disseminate. Pathogens secrete enzymes and toxins that allow their spread in tissues. Enteric pathogens induce death of intestinal epithelial cells or break their tight junctions to reach the submucosa. Various microbial toxins – such as nigericin, maitotoxin, aerolysin, gramicidin, pneumolysin, α -hemolysin, and α -toxin – form pores on the surface of host cells leading to their death. Some toxins derange the plasma membrane or the cytoskeleton, whereas others interfere with signal transduction pathways.¹¹

3. HOST DEFENSE

The host evolved means to detect pathogens' intrusion and mechanisms to restrict their growth. First-line defenses are provided by the innate immune system, which is armed by constitutive as well as

inducible defense mechanisms. In mammals, constitutive defenses include the normal microbial flora that compete with pathogens, physical barriers of the skin and internal epithelial layers, mechanical defenses of the mucus and cilia, and chemical defenses such as the acidic pH of the stomach. For a long time, innate immunity was considered nonspecific. However, in the late 1980s, Janeway proposed that detection of pathogens by PRRs was key to specific activation of immune responses.¹² Various classes of innate immunity recognition systems have been discovered. Some are soluble molecules such as mannose-binding lectins, ficolins, and collectins, whereas others are confined to cells, most notably, C-type lectins, Toll-like receptors (TLRs), nucleotide binding and oligomerization domain (Nod)-like receptors (NLRs), dsRNA helicase-like receptors (RIG-I and Mda5), and the cytoplasmic dsDNA receptors ZBP1-DAI and AIM2. PRRs recognize PAMP “signatures” displayed by microorganisms but not found on host cells. They also sense alterations in the host cellular environment arising indirectly from the infection, referred to as *danger-associated molecular patterns* (DAMPs), or alarm signals. Decrease in intracellular K^+ levels, which occurs in response to pore-forming toxins, accumulation of reactive oxygen species (ROS), or release of “alarmins” in the extracellular space are examples of alarm signals or DAMPs that activate PRRs. Alarmins are unrelated host proteins with distinct cellular functions that acquire the ability to signal tissue damage and trigger an inflammatory response when secreted in the extracellular milieu in response to infection or cell death. Most notable are antimicrobial peptides, heat shock proteins, the non-histone chromatin-binding protein HMGB1, and extracellular matrix degradation products such as hyaluronan.¹³ Activation of PRRs is transduced via a plethora of cellular proteins (kinases, ubiquitin ligases, adaptors, proteases, transcription factors), which induce a large array of interconnected and synergistic defense mechanisms aimed at killing the pathogen while preserving host cell integrity. These defenses include the production of antimicrobial peptides by epithelial cells and neutrophils, elicitation of an inflammatory response necessary for the activation of phagocytes, and the death of infected cells that, in certain instances, limits the spread of pathogens to surrounding tissues.

3.1. Antimicrobial peptides

Antimicrobial peptides (AMPs) are endogenous antibiotics that have been established as an essential part of innate immunity. They bind to a wide variety of pathogens, including Gram-negative and Gram-positive

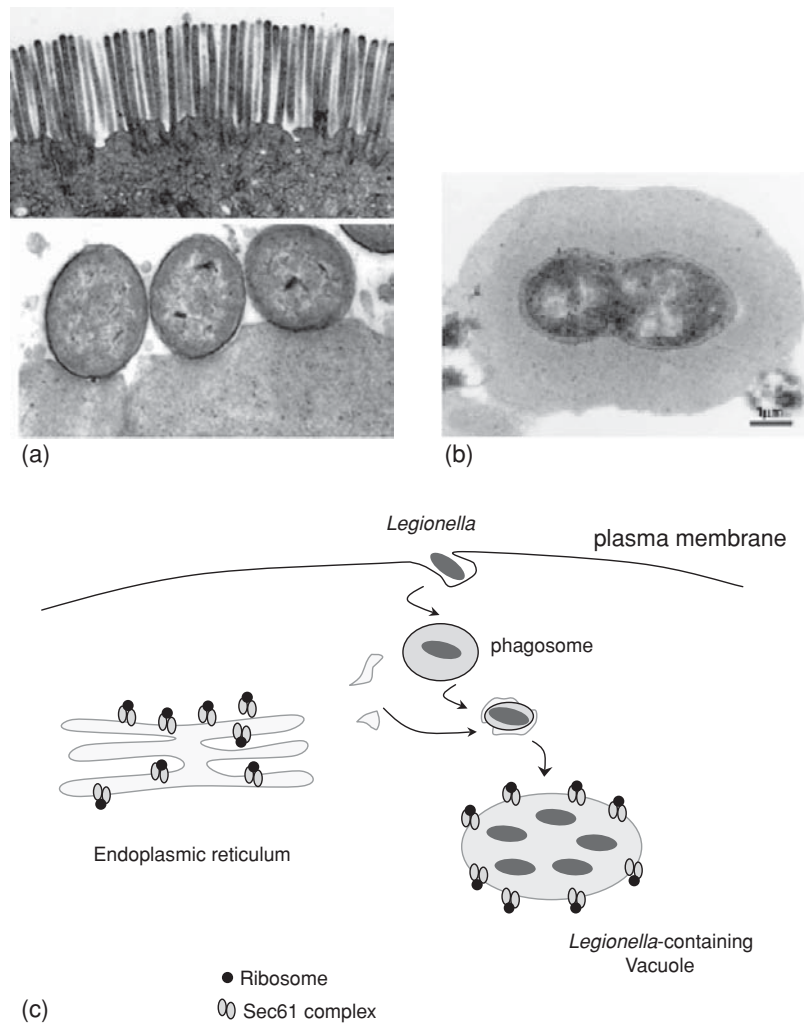


Figure 32-4. Pathogen strategies to evade immune surveillance. **(a)** Micrograph of attaching and effacing pathogens attaching tightly to colonocytes and effacing their microvilli. **(b)** Micrograph of a bacterium depicting the thick carbohydrate capsule surrounding the bacterial cell wall. **(c)** Intracellular pathogens exploit the endosomal compartment and modify the microenvironment of the phagosome to inhibit phagosome-lysosome fusion. *L. pneumophila* surrounds its phagosome with an ER-derived membrane, creating a niche for replication where it “hides” from degradation, known as the *Legionella*-containing vacuole. **(a)** and **(b)** Reprinted with permission from R. Mundy, T. T. MacDonald, G. Dougan, G. Frankel, and S. Wiles. *Citrobacter rodentium* of mice and man. *Cell Microbiol* 7 (12), 1697–706 (2005).

bacteria, fungi, and some viruses, and disrupt their cytoplasmic membrane. In addition to their direct role in killing microbes, they act as immunostimulants that modulate the inflammatory response and chemo-attract and activate antigen-presenting cells (APCs). They are small, generally cationic peptides with spaced hydrophobic and charged regions and are synthesized as prepropeptides with an N-terminal signal sequence, an anionic pro segment, and a C-terminal cationic AMP domain that gains biological activity after processing. In humans, AMPs are subgrouped into three classes based on structural characteristics: the defensins (α and β subfamilies), cathelicidins, and histatins (Figure 32-5).

Histatins (His-1 and His-3) are histidine-rich, mainly antifungal peptides found in the saliva. Defensins and cathelicidins are, on the other hand, expressed in neutrophils, keratinocytes, and epithelial cells either constitutively or on induction by bacteria and cytokines. More than 300 defensins have been identified so far in many organisms, including mammals, birds, invertebrates, and plants (<http://defensins.bii.a-star.edu.sg>). In humans, there are six α -defensins. α -defensins 1 through 4, also known as human neutrophil peptides (HNP1–4), are stored as mature peptides in neutrophil azurophilic granules and contribute to nonoxidative killing of phagocytized pathogens. Human α -defensins 5 and 6 (HD-5 and -6) are primarily produced by epithelial cells and require processing upon release. In rodents, α -defensins are termed *cryptdins*, as they are mainly found in Paneth cells of the small intestinal crypts. Cryptdins are processed by MMP7, a tissue metalloprotease also termed *matrilysin*; *MMP7*^{−/−} mice lack mature cryptdins and are susceptible to oral infection with *Salmonella*.¹⁴ Unlike in mice, matrilysin is not found in the small intestine of humans; the digestive enzyme trypsin was found to be the cleaving enzyme for HD5.¹⁵ The question of why mice and humans use different enzymes to process Paneth cells defensins remains unclear. β -defensins (hBD1, hBD2, hBD3, and hBD4) are primarily produced by

epithelial cells. The processing of β -defensins is thought to occur in a similar fashion to that of α -defensins; however, the convertases involved remain unknown. α and β defensin subfamilies are characterized by three intramolecular disulfide bonds mediated by six conserved cysteine residues and differ by the cysteine pairing and the length of peptides between the cysteines (Figure 32-5). Structurally, they are folded in a characteristic three-stranded β sheet. θ -defensins, which form a third defensin subfamily, are structurally unrelated to α and β defensins and are only found in nonhuman primates. Experiments with genetically modified mice were most informative in confirming

Group I: Linear, α -helical peptides without cysteines

LL-37

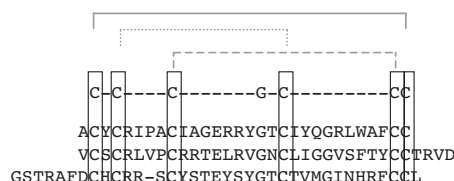
LLGDFFRKSKKEIGKEFKRIVQRIKDFLRNLPRTES

Group II: peptides with cysteines linked by disulfide bridges

Defensins

α -defensins

HNP-1
HNP-4
HD-6



β -defensins

hBD-1
hBD-2
hBD-3



Group III: Unusual high proportion of specific amino acids

Histatins

His-1
His-3

DSHEKRHHGYRRKFHEKHHSHREFPFYGDYGSNYLYDN
DSHAKRHHGYRRKFHEKHHSHRGYRSNYLYDN

Figure 32-5. Classification of antimicrobial peptides based on structural similarities. Boxes and brackets in group II delineate the cysteine residues involved in disulfide bridges.

the important role of defensins in innate immunity. For instance, deletion of mouse beta defensin-1 (mBD1) resulted in delayed clearance of *Haemophilus influenzae* from infected lungs,¹⁶ whereas transgenic expression of human intestinal defensin-5 (HD-5) in mice at physiologic levels resulted in protection against oral challenge with virulent *Salmonella typhimurium*.¹⁷ In humans, susceptibility to ileal Crohn's disease is associated with decreased production of HD-5 and HD-6 by Paneth cells,¹⁸ and genetic polymorphisms in *hBD2* are associated with Crohn's disease.¹⁹ These studies implicate defensins in the control of innate immunity to commensal microorganisms and the maintenance of intestinal homeostasis. In addition to direct microbial killing, defensins, specifically α -defensins (HNP1-3)

and synthetic θ -defensins (retrocy-clins), were reported to neutralize the enzymatic activity of certain bacterial toxins, namely that of *Bacillus anthracis*'s lethal toxin (LT), diphtheria toxin (DT), and *Pseudomonas* endotoxin A (ETA), protecting from toxin-associated lethality both in vitro and in vivo.²⁰ The only cathelicidin found in humans is LL-37/hCAP18. Its murine ortholog cathelicidin-related AMP (CRAMP) has been shown in vivo to exert protective effects during bacterial infections.²¹ Consistent with previous observations suggesting anti-LPS activities by certain AMPs and protective effects from lethal endotoxemia, synthetic LL-37 derivatives lacking bactericidal activity were shown to exert protective immunomodulatory activities in monocytes and macrophages. Specifically, one peptide termed innate defense regulator peptide (IDR-1) dampened the expression of proinflammatory cytokines in response to LPS while inducing chemokine levels and was efficacious in countering infections without obvious toxicities.²²

In mammals, PRR signaling pathways, including those downstream of TLRs and NLRs, appear essential for the expression of AMPs, specifically β -defensins. In this aspect, there is a striking parallel in the regulation of AMP production between mammals and insects.²³ Insects are notorious for

their resistance to infections. Their immune response depends heavily on the production of AMPs by the fat body, which is a functional equivalent of the mammalian liver. In *Drosophila melanogaster*, two distinct signaling pathways, referred to as Toll and Imd pathways, regulate AMP production. Within these pathways, three nuclear factor kappa B (NF- κ B) proteins – namely Relish, Dorsal, and Dif – are central to the transcriptional induction of AMP genes. Fungi and Gram-positive bacteria largely activate the Toll pathway. Unlike mammalian TLRs, *Drosophila* Toll is not a PRR. It is activated by the cytokine spätzle, which leads to the production of the antifungal AMP *Drosomycin*, which defends against fungal and Gram-positive bacterial infections. In contrast, the immune deficiency (Imd) pathway is

activated by Gram-negative bacteria and through Relish induces the production of a number of different AMPs, including *Diptericin*. Whereas the Toll pathway shares significant homology with the TLR and interleukin (IL)-1R pathways in mammals, the Imd pathway is related to the mammalian tumor necrosis factor receptor (TNFR) and NOD pathways (Figure 32-6). This striking conservation between the fly and mammalian pathways points to a common ancestry of these immune mechanisms.

3.2. PRRs and inflammation

3.2.1. TLRs

The innate immune system was extensively studied in *Drosophila* with the aim of elucidating how fruit flies that lack adaptive immunity responded to infectious pathogens. The discovery of *Drosophila* Toll was followed by the cloning of a human homolog and its characterization as a PRR able to stimulate the NF- κ B pathway.²⁴ So far, 12 members of the Toll family have been identified in mammals and are referred to as the Toll-like receptors (TLRs). All known TLRs are type I transmembrane proteins consisting of an extracellular domain with leucine-rich repeats (LRRs) responsible for ligand detection and a cytoplasmic Toll/IL-1R homology (TIR) domain essential for initiating signaling²⁵ (Figure 32-7). TLRs function as homo- or heterodimers and recognize a broad range of microbial components.²⁶ Mouse genetics studies and investigation of TLR knockout mouse phenotypes demonstrated the essential role of these receptors in pathogen recognition. Mice with a point mutation in *Tlr4* are hyporesponsive to LPS.²⁷ *TLR3*^{-/-} mice fail to recognize viral double-stranded RNA (dsRNA) and are defective in stimulating inflammatory and type I interferon (IFN) responses.²⁸ By associating with other TLRs, TLR2 responds to a variety of bacterial molecules that include peptidoglycan, lipoproteins, and lipopeptides. TLR5 recognizes bacterial flagellin,⁸ TLR7 responds to synthetic compounds with potent antiviral and antitumor activities such as resiquimod (R848), and TLR9 senses CpG motifs in bacterial and viral DNAs.²⁹ TLRs share common signaling determinants with IL-1R family members; their TIR domains interact with TIR-containing adaptors, including MyD88, TIRAP/MAL, TRIF, and TRAM.³⁰ With the exception of TLR3, all TLRs signal through MyD88. TIRAP/MAL, TRIF, and TRAM are more specialized. TRIF is engaged in response to viral PAMPs downstream of TLR3 and TLR4, MAL interacts with TLR2 and TLR4, and

TRAM binds TRIF in the TLR4 complex. The multistep signaling cascade induced by TLR activation results in the production of antimicrobial effectors by means of the NF- κ B, mitogen-activated protein kinases (MAPKs), and interferon regulatory factor IRF 5/3/7 pathways³¹ (Figure 32-7).

3.2.2. NLRs

NLRs are evolutionarily related to disease resistance or “R” proteins in plants. These are cytosolic proteins that contain a nucleotide-binding site (NBS) and leucine-rich repeats (LRRs) and are often involved in the recognition of PAMPs and pathogen-induced host danger signals. Upon activation, R proteins elicit a hypersensitive or guard response, which induces antimicrobial proteins, cell wall modification, and programmed cell death.³² Although the defense systems in animals and plants evolved under selective pressure imposed by distinct infectious pathogens, they stayed remarkably conserved across the two kingdoms. Another level of similarity between the two systems is the role that Hsp90 and SGT1 play in stabilizing the NBS-LRR/NLR proteins and maintaining their conformation in an auto-repressed but activation-competent state.³³

The first described mammalian NLR, Nod1, was identified in a screen aimed at finding Apaf-1–related proteins.³⁴ Similar to Apaf-1, Nod1 possesses an N-terminal CARD domain, a central nucleotide binding and oligomerization domain (Nod), and a C-terminal agonist-binding domain. The latter is a WD-40 repeat domain in Apaf-1 that binds cytochrome c, whereas it is an LRR domain in Nod1 that senses bacterial peptidoglycan derivatives.³⁵ In response to agonist sensing, NLRs oligomerize and activate inflammatory effectors. To date, there are 22 NLRs, including 14 Nlrp proteins (Nlrp1–14), 5 Nods, Ipaf, Naip5, and CIITA. Comparison of the NLRs reveals both structural and functional differences. The N-terminal effector domain is variable among NLRs; it is a CARD in Nod1 and Ipaf, two CARDS in Nod2, three BIRs in Naip, and a pyrin domain (PYD) in Nlrp1-14 (Figure 32-8).

3.2.3. The Nod signalosome

Despite the presence of a CARD in Nod1 and Nod2, these proteins do not engage caspases directly. Instead, they interact with a CARD-containing kinase known as RIP2. This assembles a Nod signalosome that recruits TRAF proteins, cellular inhibitor of apoptosis proteins cIAP1 and cIAP2, TAK1, TAB1 and TAB2, needed to activate the

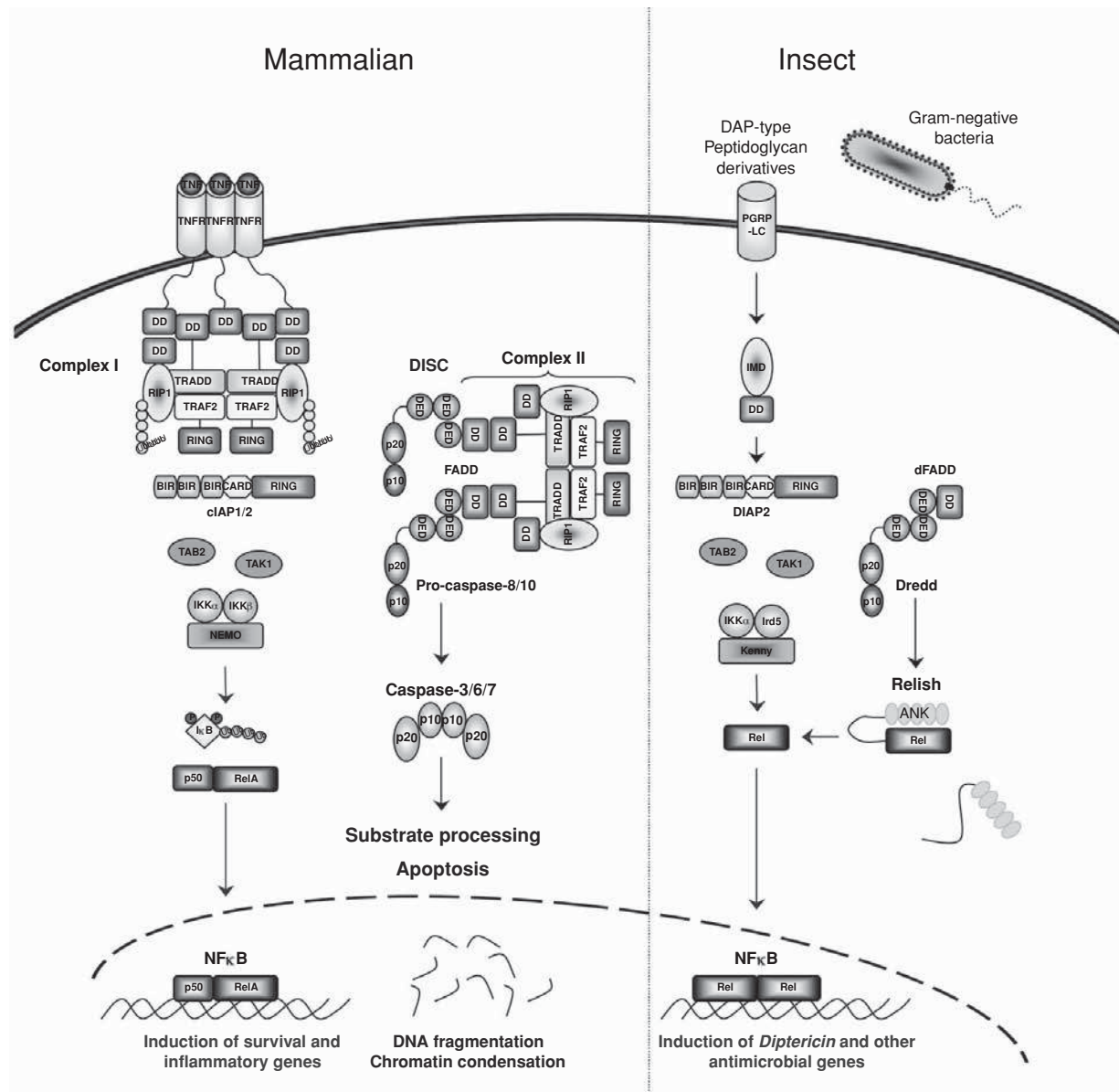


Figure 32-6. Schematic representation of the mammalian TNF and insect Imd pathways. In mammals, TNF induces an inflammatory response mediated via the assembly of a signaling complex at the TNF receptor level, known as complex I. Through the recruitment of the adaptor TRADD (TNF receptor associated protein with a death domain [DD]), the kinase RIP-1 (receptor-interacting protein 1) and the E3 ubiquitin ligases, TRAF2 (TNF receptor-associated factor 2), cIAP1 and cIAP 2 (cellular inhibitor of apoptosis proteins), the signal is transduced to the kinases TAK1, TAB2, and IKKs, resulting in the activation of the NF- κ B and MAPK inflammatory pathways. In conditions promoting cell death, TRADD, TRAF2, and RIP-1 dissociate from complex I and recruit the DISC (death-inducing signaling complex), which is composed of FADD and caspases-8/10, to form complex II, which is necessary to oligomerize and activate the caspases and initiate the extrinsic apoptosis program. In *Drosophila melanogaster*, the Imd (immune deficiency) pathway is engaged in response to infection with Gram-negative bacteria. Peptidoglycan derivatives from the bacterial cell wall activate PGRP-LC receptors on the plasma membrane of fat body cells, which transduce the signal to the NF- κ B protein Relish, which in turn induces the expression of antimicrobial peptide genes such as *Diptericin*. The fly Imd signal transduction pathway shares similarities with the mammalian TNF pathway. Common effectors include a RIP1-like adaptor with a death domain (Imd), an IAP protein (DIAP2), the kinases TAK1 and TAB2, IKK β (ird5), IKK γ (Kenny), and NF- κ B (Relish). In addition, dFADD and Dredd (the homolog of caspase-8) play an essential role in the activation of Relish, a homolog of mammalian NF- κ B p105. One main difference between the two systems is that Dredd cleaves the inhibitory ankyrin domain of Relish, freeing its Rel transcription domain to translocate to the nucleus and activate target genes. See Color Plate 38.

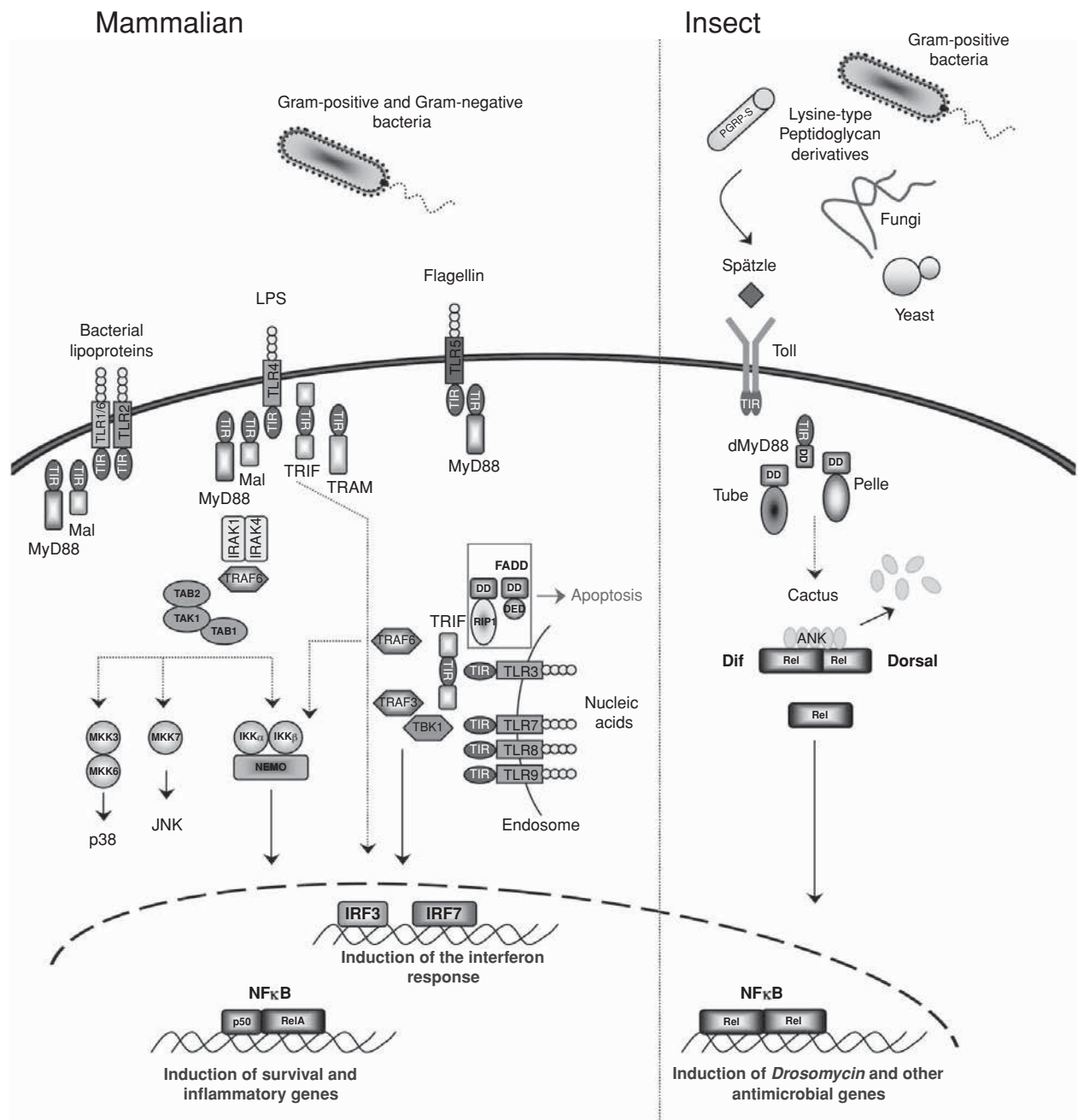


Figure 32-7. A schematic representation of the mammalian TLR and insect Toll pathways. One important difference between the two systems is that, unlike TLRs, insect Toll is not a pattern recognition receptor (PRR). In response to infections with Gram-positive bacteria, fungi, and yeast, soluble PRRs are activated in the hemolymph, resulting in the processing of Spätzle, a cytokine-like molecule that engages Toll. Downstream of Toll or TLR activation, there is a certain degree of similarity in the signal transduction pathways. See Color Plate 39.

proinflammatory NF- κ B and MAPK pathways. Recently, a protein termed CARD9 has been reported as a new addition to the Nod signalosome. CARD9 interacts with the Nod-Rip2 complex and selectively mediates activation of the MAPK pathway downstream of Nod stimulation⁽³⁶⁾ and references therein). Similar to RIP1's

activation at the TNFR level, polyubiquitination of RIP2 by cIAP1 and cIAP2 is key to the transduction of the signal from Nod proteins to NF- κ B and MAPK.³⁷ The Nod signalosome is negatively regulated by caspase-12, which was shown to interact with RIP2 and inhibit Nod signaling by blocking RIP2 ubiquitination.³⁸

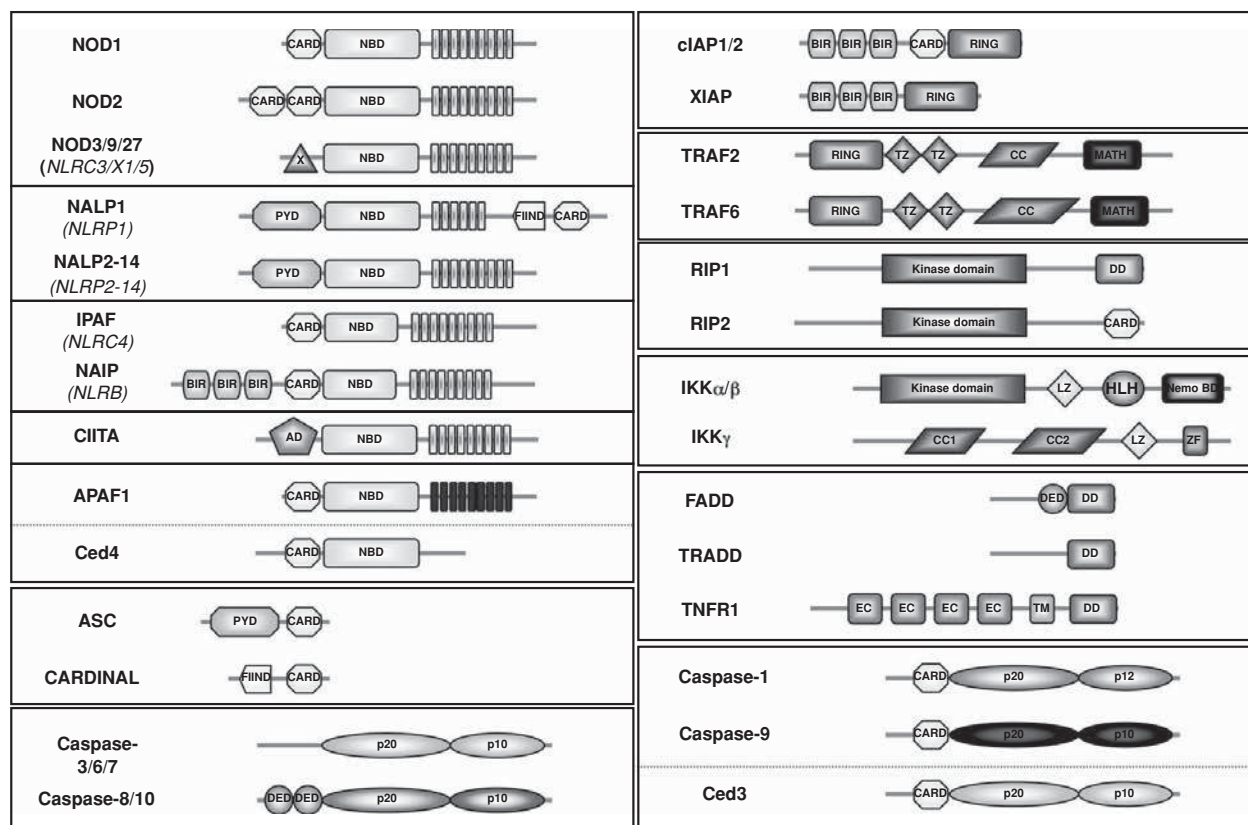


Figure 32-8. Effectors of inflammation. The domain structures are shown. AD, transcriptional activation domain; CC, coiled-coil; EC, extracellular domain; FIIND, domain with function to find; HLH, helix-loop-helix domain; LZ, leucine zipper domain; MATH, meprin- and TRAF-homology domain; NBD, nucleotide-binding domain; PDZ, protein domain named after the initial letters of PSD-95, Dlg, and ZO-1; TM, transmembrane domain; TZ, TRAF-type zinc-finger domain; ZF, zinc finger domain. See Color Plate 40.

3.2.4. The inflammasome

Unlike Nod proteins that signal through RIP2, Nlrp proteins, Ipaf, and Naip recruit and activate inflammatory caspases, predominantly caspase-1, in a multiprotein complex known as the inflammasome.^{39,40} The activation of caspase-1 results in the processing and maturation of the cytokines IL-1 β and IL-18. IL-1 β and IL-18 exert various effects on different tissues, which result in the induction of fever, anorexia, fatigue, fat catabolism, secretion of acute-phase proteins, and activation of immune cells, leading to the release of other cytokines and chemokines.⁴¹ The recruitment of caspase-1 and its activation in the inflammasome mirrors that of caspase-9 in the apoptosome during mitochondrial apoptosis (Figure 32-9). Structurally, the Nlrp1 inflammasome observed by cryoelectron microscopy also seems to share with the apoptosome its oligomeric nature.⁴² Binding of caspases to NLRs occurs through a CARD–CARD interaction, which is either direct, as in the case of Ipaf binding to caspase-1⁴³

and Nlrp1 binding to caspase-5,⁴⁴ or indirect, as in the case of PYD-containing Nlrp proteins.⁴⁴ Adaptor molecules mediate the association between caspases and Nlrp proteins.⁴⁰ Two adaptors have been identified, namely a PYD–CARD-containing adaptor termed Apoptosis-associated Speck-like protein containing a Caspase-recruitment domain (ASC) (also known as PYCARD), and Cardinal, a protein with homology to the C-terminal region of Nlrp1. Among the inflammatory caspases, caspase-1 is universal to all inflammasomes and is the sole “effector” of cytokine processing. Caspase-5 is recruited to the Nlrp1 inflammasome,⁴⁴ where it acts as a caspase-1 cofactor, but does not appear to be involved in the Nlrp3, Ipaf, and Naip5 complexes. Caspase-11, the murine ortholog of caspase-5, has also been suggested to act in vivo as an essential activator of caspase-1. In support of this, caspase-11-deficient mice were shown to be incapable of producing IL-1 β and IL-18 in response to LPS stimulation.⁴⁵ Nonetheless, it seems that the requirement for caspase-11 is not absolute but is restricted to certain stimuli, as caspase-1

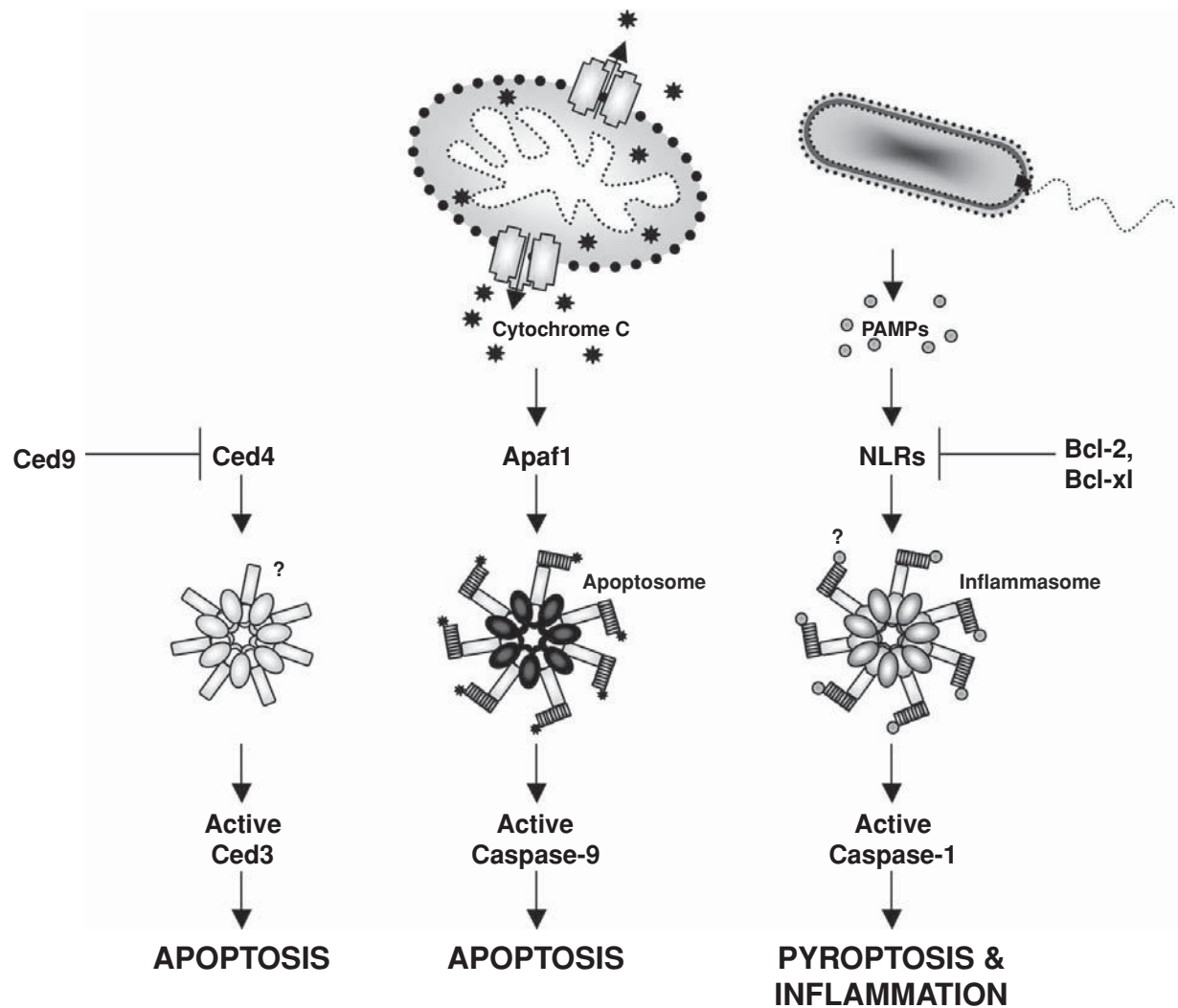


Figure 32-9. A parallel between mitochondrial apoptosis and NLR innate immunity pathways. The apoptosome is scaffolded by the protein Apaf-1 and assembles in response to cytochrome c release from the mitochondria during intrinsic apoptosis to activate caspase-9. NLR proteins that share a conserved structure with Apaf-1 scaffold the inflammasome. During bacterial infection, bacterial products (PAMPs) or host-derived alarm signals arising during the infection stimulate the assembly of the inflammasome into an oligomeric complex that recruits and activates caspase-1. Activation of caspase-1 leads to pyroptosis and inflammation. A similar pathway exists in the nematode *Caenorhabditis elegans*, where Ced4, a homolog of Apaf1, oligomerizes with the caspase Ced3 to induce apoptosis. With the exception of the apoptosome, which is a heptameric oligomer, the number of proteins in the inflammasome and CED4 oligomers is hypothetical (question marks). It is also unknown whether PAMPs bind directly to NLRs. See Color Plate 41.

could be normally activated in the absence of caspase-11 in response to *Listeria* infection.⁴⁶ Caspase-12 has been recently shown to act as an inhibitor of caspase-1.⁴⁷ The extent of caspase-1 activation appears to vary among inflammasomes. Ipaf, as compared with NLRp3, activates caspase-1 more robustly. This results in rapid caspase-1-dependent cell death, or pyroptosis (discussed below in 3.3.2), in addition to cytokine maturation and release. It has been recently proposed that, in response to K⁺ efflux, cells assemble one large ASC oligomer, or speck, per cell. Termed the pyroptosome, this platform is hypothesized to recruit most of the cellular caspase-1

off NLRs, leading to increased caspase-1 activation and cell death.⁴⁸ In vivo findings, however, do not implicate ASC in pathogen-induced pyroptosis, as cell death proceeds normally in ASC-deficient cells, whereas it is inhibited in *caspase-1*^{-/-} or *Ipaf*^{-/-} cells. Both Ipaf and ASC are fully required for IL-1 β production in response to *Salmonella*, *Pseudomonas*, or *Legionella* infection, yet only Ipaf-deficient macrophages are fully resistant to pyroptosis induced by these pathogens, whereas ASC-deficient cells are only partially protected.^{43,49,50,51}

The presence of LRRs in NLR proteins is thought to mediate recognition of PAMPs by these cytosolic

receptors. However, for this recognition to be possible, PAMPs must reach the cytosol. Various mechanisms of PAMP delivery to the cytosol have been described that result in inflammasome activation. For instance, the facultative intracellular Gram-positive bacteria *Listeria monocytogenes* escapes the phagosome into the host cytosol, its replicative niche, via the actions of a pore-forming toxin named listeriolysin O (LLO).⁵² In the cytosol, *Listeria* is sensed by multiple inflammasomes.⁵³ Bacterial secretion systems have also been shown to deliver PAMPs to the cytosol. *Salmonella typhimurium* SipB, thought previously to activate caspase-1 through direct binding,⁵⁴ mediates the activation of the Ipaf inflammasome indirectly by facilitating the delivery of bacterial flagellin into the cytosol through a type III secretion system.⁵⁵ Flagellin, from *Legionella pneumophila*⁴⁹ and *Pseudomonas aeruginosa*, is similarly delivered via bacterial secretion systems and sensed by the Ipaf inflammasome, leading to caspase-1 activation.^{50,51,56} This mechanism of delivery to the cytosol is not exclusive to flagellin, as bacterial secretion systems have been also proposed to translocate peptidoglycan derivatives from extracellular bacteria, such as *Helicobacter pylori*⁵⁷ and EHEC,³⁸ resulting in the activation of Nod signaling. An alternative mechanism through which bacterial products could reach the cytosol is through the pannexin pore.^{58,59} Pannexin is a hemichannel that gets recruited to the cell membrane in response to activation of the P₂X₇ receptor by adenosine triphosphate and opening of K⁺ channels.⁶⁰ This leads to the gradual formation of a larger pore that has been shown to mediate the intracellular delivery of muramyl dipeptide, leading to caspase-1 activation.^{59,61}

Pore formation in the plasma membrane by various microbial toxins such as nigericin, maitotoxin,⁵³ and aerolysin⁶² also results in inflammasome activation, although independently of bacterial product translocation into the cytosol. It is proposed that K⁺ efflux from the cell through these pores or through K⁺ channels is sensed as a danger signal by the Nlrp3 inflammasome.⁵³ Unlike Ipaf activation, which seems to be specific to flagellin and bacterial type III secretion systems, the Nlrp3 inflammasome is activated by a wide array of triggers, some derived from pathogens, but others derived from the host. The list of Nlrp3 activators is expanding steadily and includes thus far toxins (nigericin, maitotoxin, aerolysin, listeriolysin O, gramicidin, and α -toxin),^{53,62,63} K⁺ efflux,⁶⁴ UVB,⁶⁵ asbestos and silica,⁶⁶ viral and bacterial RNA,⁶⁷ uric acid crystals,⁶⁸ and microbial and host DNA.⁶⁹ The mechanism by which the Nlrp3 inflammasome responds to all these divergent activators possibly relies on the generation of a downstream signal that itself feeds down on the activation of the

inflammasome. Perturbation of the intracellular ionic milieu and/or production of intermediate metabolites, such as ROS, have been proposed to be the link to the Nlrp3 inflammasome.^{64,70}

3.3. Cell death

Cell death is a key process that tailors host-pathogen interactions and is the most common outcome during infections. The death of an infected cell is often-times concomitant with the death of the infecting agent and can promote efficient pathogen clearance. Destruction of infected tissues may also eliminate a pathogenic niche, thereby hampering microorganism replication and dissemination.

Pathogens have devised strategies to inhibit cell death for a successful infection (Figure 32-10). Conversely, certain pathogens induce death of immune cells as a means to subvert normal host defense mechanisms and of epithelial and endothelial cells for invasion to deeper layers of an organ and the bloodstream. Killing of phagocytes impairs pathogen clearance and is detrimental to the host. By producing cytotoxic pore-forming exotoxins, bacteria such as *Bacillus anthracis*,⁷¹ *Actinobacillus actinomycetemcomitans*,⁷² and *Pseudomonas aeruginosa*⁷³ kill macrophages before they themselves are phagocytosed and destroyed. Similarly, *Bordetella pertussis* adenylate cyclase hemolysin secretion during the early stages of infection may allow for successful colonization of alveolar tissue by eliminating the local macrophage population.⁷⁴

3.3.1. Apoptosis and pathogen clearance

The mechanism of pathogen-induced cell death often involves the modulation of the apoptotic response. Apoptosis of infected cells is thought to dampen pathogen spread yet protect the integrity of infected tissues. Several pathogens induce apoptosis by interfering with the NF- κ B and/or MAPK cell survival pathways. For example, *Salmonella* AvrA and *Yersinia* YopJ proteins inhibit NF- κ B activation, whereas *Bacillus anthracis*'s protease lethal factor (LF), a component of its LT, targets MKK6 to dampen MAPK signaling (Figure 32-10). Other pathogens have devised strategies to inhibit cell death for a successful infection. For obligate intracellular organisms such as *Rickettsia rickettsii*, a viable host cell is required for bacteria to replicate and thrive. By stimulating NF- κ B signaling, *Rickettsia* prevents host cell death and continues to replicate unabated. Another intracellular pathogen, *Chlamydiae* spp., also protects infected cells from death during the early invasive stages of the disease, presumably by blocking cytochrome c release from the mitochondria. Genetic studies provide

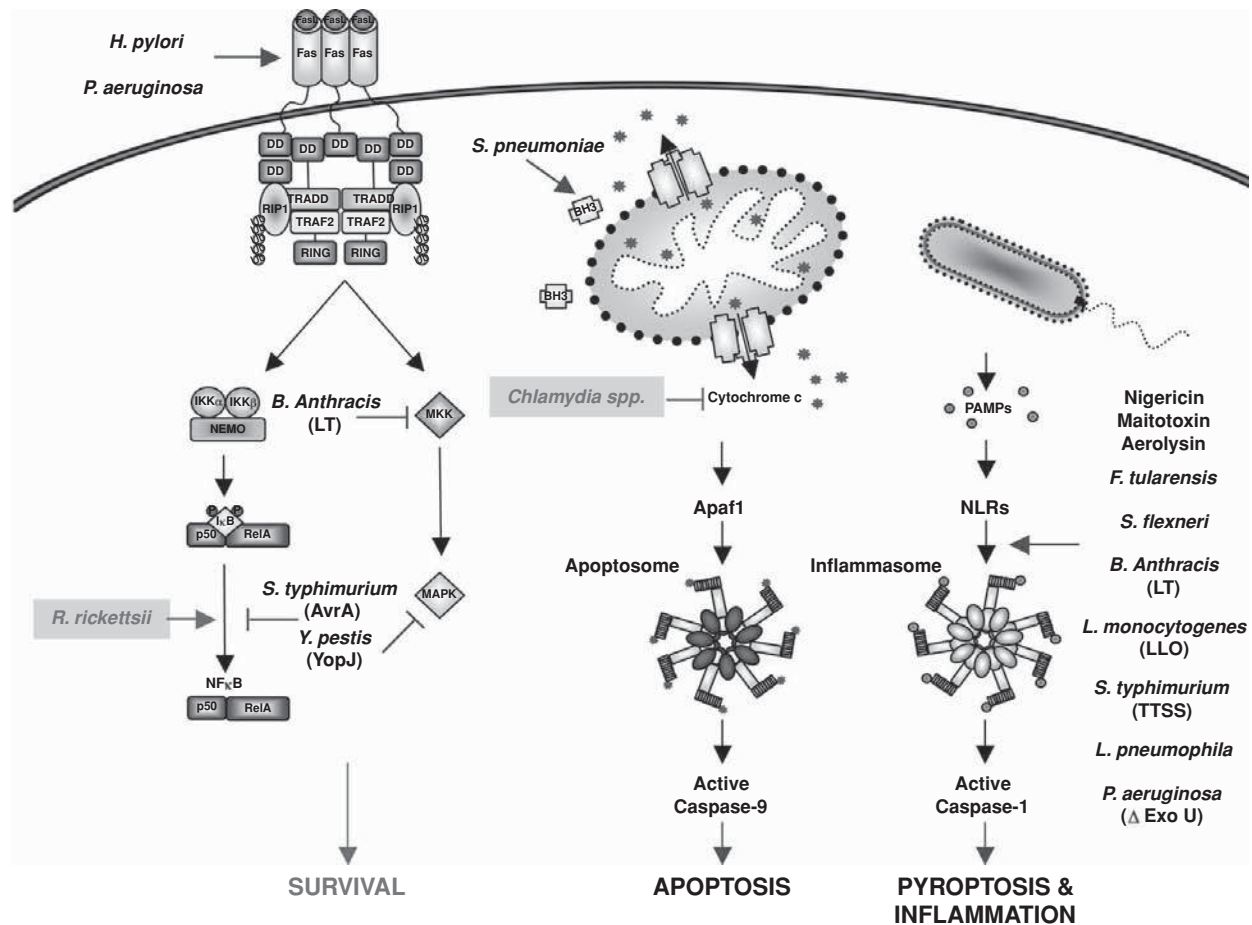


Figure 32-10. Modulation of cell survival/death pathways by microbial effectors. Pathogens and pathogen-derived molecules that activate or block survival or death signaling are represented in green or red, respectively. ExoU, exoenzyme U; LLO, listeriolysin O; LT, lethal toxin; TTSS, type III secretion system; YopJ, *Yersinia* outer coat protein J. See Color Plate 42.

the most stringent evidence of apoptosis induction by a pathogen and allow the evaluation of the effects of cell death on host resistance to infection. An excellent example is that of alveolar macrophage apoptosis by pneumococci. Over-expression of the antiapoptotic protein Mcl-1 in a transgenic mouse model blocks apoptosis and renders mice susceptible to infection, indicating that apoptosis is indeed triggered by pneumococci and that it is protective for the host. On the other hand, apoptosis could be pathogenic. During sepsis, lymphocytes are depleted by apoptosis, which leads to anergy and immunosuppression. In an experimental model of sepsis, inhibition of apoptosis by selective caspase-3 inhibitors or by Bcl-2 over-expression was shown to lower sepsis-related mortality.

3.3.2. Pyroptosis

Cell death triggered by intracellular pathogens such as *Salmonella* and *Shigella* was initially thought to occur by apoptosis, a death executed by apoptotic caspases.

Later, however, caspase-1, a prototypical inflammatory caspase, was demonstrated to be the central effector of this cell death. This is evidenced by the resistance of caspase-1-deficient macrophages to the quick death induced by these pathogens. Unlike apoptosis, caspase-1-dependent cell death is accompanied by the release of proinflammatory mediators from the cell. The term *pyroptosis* has been proposed to describe this proinflammatory programmed cell death (*pyro* relating to fire or fever and *ptosis* denoting falling). Caspase-1 is essential for the processing, maturation, and release of the cytokines IL-1 β and IL-18. It has also been recently proposed to act as a regulator of unconventional protein secretion,⁷⁵ a process that might mediate the release of “danger” proteins to the extracellular milieu. These functions of caspase-1 contribute to the proinflammatory nature of pyroptosis (Figure 32-11). The release of inflammatory factors by pyroptotic cells is, however, not what kills the cell. The mechanisms by which caspase-1 executes cell death have been recently addressed. Multiple direct caspase-1

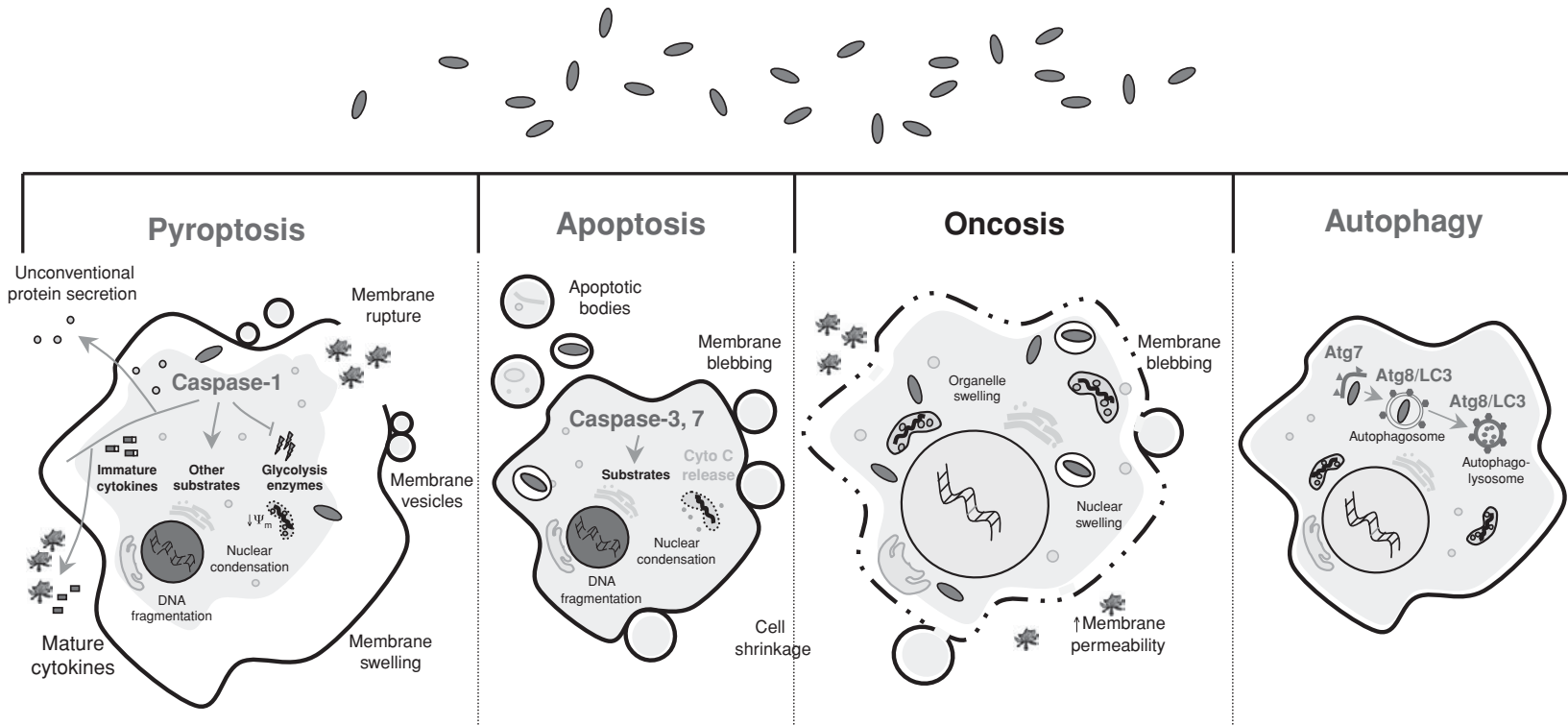


Figure 32-11. Pathogen-induced host cell death. Several forms of host cell death have been described during infection. The type of death the cell undergoes depends on the nature of the pathogen, pathogen load, and site of infection. Pyroptotic, apoptotic, autophagic, or oncototic cells display a distinct set of morphological and biochemical characteristics, some of which are shared. Whereas apoptosis and autophagy do not induce inflammation, cytokine release and escape of cytoplasmic content during pyroptosis or oncosis are highly inflammatory events. Pathogens are depicted as red ovals. During pyroptosis, pathogens (or pathogenic products) in the cytosol are detected by caspase-1-activating inflammasomes. During apoptosis, pathogens are contained within apoptotic bodies and digested in the lysosomes of phagocytes that engulf apoptotic cells. During autophagy, pathogens are surrounded by autophagosomes and delivered to the lysosomes via autophagosome-lysosome fusion. Although apoptosis, pyroptosis, and autophagy are generally beneficial to the host, oncosis favors pathogen dissemination. See Color Plate 43.

substrates involved in cytoskeletal maintenance and energy metabolism have been identified, suggesting that, analogous to apoptosis, cleavage of these substrates is what mediates the distinct morphology of pyroptotic cells and eventually leads to their death. Another hypothesis stipulates that pyroptotic cells lyse as a result of the formation of membrane pores, which cause loss of ionic equilibrium, water influx, swelling, and membrane rupture with release of inflammatory intracellular contents. Caspase-1-deficient mice are susceptible to infection by various pathogens. The susceptibility of these mice could be attributed to either lack of a proper innate immune response in the absence of mature IL-1 β and IL-18 or to a defect in macrophage cell death. Without being able to experimentally tease apart the two different roles of caspase-1 in inflammation and cell death, it is not possible to make conclusions about the role of pyroptosis in pathogen clearance.

3.2.3. Caspase-independent cell death

Cells possess several mechanisms to execute cell death. Several of these are caspase-independent and have been described for infected cells. For instance, although caspase-1-deficient macrophages are initially resistant to death by many bacteria, they eventually succumb in a caspase-independent fashion. Similarly, in the case of *Mycobacterium tuberculosis*, infected macrophages undergo apoptosis, but inhibition of caspases does not prevent cell death. A serine protease inhibitor appears to block this caspase-independent death.⁷⁶ Moreover, at high multiplicity of infection (MOI), *M. tuberculosis* induces a caspase-independent cell death that is not observed at low MOI.⁷⁷ In the case of *Shigella*, the primary death mode is pyroptosis, induced through the Ipaf-caspase-1 inflammasome.⁷⁸ However, at higher MOI, *Shigella* induces a caspase-1-independent form of cell death, termed *pyronecrosis*.⁷⁹ Disease-associated cryopyrin appears to trigger this death mode as well, which is independent of caspase-1 but presumably requires cathepsin B.⁷⁹ The IPAF-caspase-1 inflammasome has been recently shown to be essential for the initiation of a proper innate immune response to *Pseudomonas aeruginosa*.^{50,56} Virulent *P. aeruginosa* isolates that evade the immune response express the effector protein exoenzyme U (ExoU). Interestingly, ExoU blocks caspase-1 activity and prevents the production of proinflammatory cytokines. However, despite inhibiting caspase-1, ExoU-expressing *P. aeruginosa* very efficiently killed macrophages.⁵⁰ Therefore, it appears that caspase-independent death occurs as a “back-up” strategy or when cells are overwhelmed with a high bacterial

load. Whether it performs a physiologic function similar to that of apoptosis or pyroptosis remains open for debate.

3.2.4. Autophagy and autophagic cell death

Autophagy can be triggered in infected host cells, presumably as a host defense mechanism for eliminating pathogens without disposing of the entire cell.⁸⁰ In a situation in which normal phagolysosomal maturation is blocked, such as during *M. tuberculosis* infection, the initiation of autophagy can overcome this inhibition and result in bacterial degradation.⁸¹ *Listeria monocytogenes*, *Salmonella enterica*, *Francisella tularensis*, and the parasite *Toxoplasma gondii* have also been shown to be targeted by autophagy.^{81,82,83} To demonstrate the importance of autophagy in intracellular pathogen clearance, Nakagawa and colleagues⁸² have shown the effective elimination of the pathogenic group A *Streptococcus* (GAS) within nonphagocytic cells via autophagy. *Atg5*^{−/−} cells allowed GAS survival, replication, and subsequent release to the surroundings, indicating that autophagy is protective for the host. Conversely, autophagosome formation may support the replication of poliovirus, rhinovirus, and *Legionella pneumophila* in host cells, as these microorganisms have devised ways to subvert the autophagosome machinery to their own benefits.⁸⁴

The type and outcome of pathogen-induced cell death depend on the nature of the infection itself (Figure 32-11). A wide variety of microorganisms have evolved mechanisms to modulate host cell death and to use a step in cell death to their advantage. Characterization of pathogen-induced cell death not only gives insight into disease pathogenesis, but also helps in the understanding of the basic mechanisms of the different cell death modalities under normal physiologic conditions.

4. CONCLUSIONS

Sequencing of the human genome and that of various pathogens, together with advances in molecular biology and genetic manipulation techniques, have resulted in an outburst of discoveries in the host–pathogen field. However, despite past progress in areas such as vaccination, hygiene, and antimicrobials, the extent and impact of infectious diseases on both developed and developing nations is regaining added prominence in the 21st century, as evidenced, for example, by SARS and West Nile virus outbreaks. Numerous circumstances, including rapid societal and technological changes, have

influenced the emergence and re-emergence of infectious diseases. Many of these factors, including aging populations, a heavier chronic disease burden, therapeutic suppression of host defenses, changing behaviors, and strong antibiotic selection pressure, act by increasing human susceptibility to infection. Further work is therefore required to understand the different measures microbial pathogens employ to infect the host and to discover means to strengthen our response and overcome the infection.

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33

**Programmed Cell Death in the Yeast
*Saccharomyces cerevisiae***

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The yeast *Saccharomyces cerevisiae* is one of the most studied model systems for molecular and cellular biology. In 1996, it became the first eukaryotic organism to have a completely sequenced genome (Dujon, 1996; Goffeau et al., 1996), which led to a number of valuable and widely accessed databases. Among its features is the short generation time (usually 90–120 minutes) and the ability to grow at various temperatures in relatively inexpensive media. Moreover, many of its genes are well characterized, thanks in part to its amenability to modifications such as gene disruption, gene marking, mutations, or gene-dosage modifications. Because of these advantageous features, it has become the model organism of choice for many investigators in fields ranging from basic biology to biomedical research.

One of the most studied subjects of the past decade is the programmed cell death, or apoptosis, a highly coordinated cellular suicide program that is crucial for maintenance of health and tissue function and the focus of this book. Apoptosis is a very complex phenomenon that is relatively well understood and is implicated in diseases ranging from cancer to neurodegenerative disorders. Many of the apoptosis-related genes were discovered and studied in model organisms, primarily in *Drosophila melanogaster* and *Caenorhabditis elegans*. Because for the unicellular yeast programmed cell death implies a highly controversial organismal suicide, the research on yeast apoptosis started late. However, during the last 10 years, many studies reported on the existence of programmed cell death in yeast and, at present, at least the possibility that a single cell organism can undergo a death program is becoming widely accepted. It is still not yet widely accepted that yeast are capable of undergoing apoptosis as strictly defined in animal species, in part because these simple eukaryotes lack caspases. For purposes of this chapter, however, we nevertheless

refer to the apoptosis-like cell death process of yeast as apoptosis.

Apoptosis in yeast can be induced by a variety of compounds and conditions, including hydrogen peroxide and acetic acid, amiodarone, hyperosmotic stress, and aging. Genetics studies also contributed to the understanding of the mechanisms of cell death in yeast by revealing the role of various genes in a form of cell death accompanied by the appearance of features of mammalian apoptotic cells. Some of these mutations are in analogues of crucial components of the apoptotic cascade in mammals such as yeast apoptosis-inducing factor (*AIF*), metacaspase (*YCA1*), an inhibitor of apoptosis protein (*BIR1*), OMI/Htr2A (nuclear mediator of apoptosis; *NMA111*), and DJ-1 (HSP31), as well as a nuclease (TAT-D) that is apparently involved in DNA degradation during apoptosis.

Likewise, some genes involved in yeast cell death have been confirmed as apoptotic regulators in metazoans. Although the yeast genome does not appear to contain obvious orthologs of the mammalian Bax and Bcl family genes, it was shown that cell death in *S. cerevisiae* can be induced by the expression of Bax, and it is accompanied by typical features of apoptosis, such as externalization of phosphatidylserine at the surface of the cytoplasmic membrane, cytochrome c release, membrane blebbing, chromatin condensation and margination, and DNA cleavage. The simultaneous expression of Bcl-xL or Bcl-2 prevents these effects and cell death. More importantly, Bax-mediated cell death in yeast, as in mammalian cells, involves mitochondrial dysfunction, leading to the release of *cyt c* and apoptosis, supporting the hypothesis that Bax can function in yeast in a way analogous to its role in mammals. Therefore, mitochondria play a central role in both metazoan and yeast apoptosis. In fact, in addition to cytochrome c,

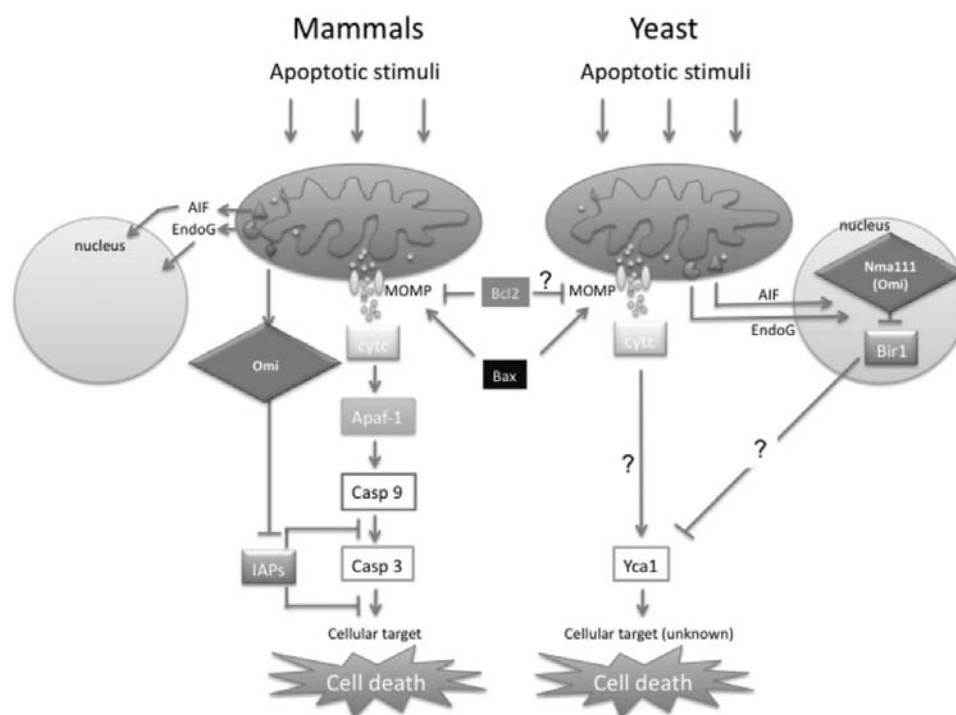


Figure 33-1. Components of apoptotic pathways are conserved from yeast to mammals. The conserved proteins involved in this pathway include cytochrome c, Aif, EndoG, caspase, Omi/HtrA, and IAP.

mitochondria represent the home of many proapoptotic molecules (i.e., AIF, endonuclease G) and are the site of early morphological changes that occur during programmed cell death. Fragmentation of tubular mitochondrial network is visible in yeast cells treated with acetic acid, H_2O_2 , amiodarone, or ethanol. These changes are similar to the thread-to-grain transition observed in mammalian mitochondria during apoptosis. These similarities between the mammalian and yeast apoptotic pathways are represented in Figure 33-1.

1. PHENOTYPE AND ASSAYS OF YEAST APOPTOSIS

Various techniques, such as dyes based on metabolic activity including MTT or phloxine B, are available to measure the viability of yeast cells (Cannon et al., 1986; Teparic et al., 2004), but the counting of colonies generated by viable individual cells is the simplest and preferred method. In fact, in contrast to cultured mammalian cells, viable individual yeast cells reliably give rise to colonies. A defined number of viable cells (usually <500) are plated on complete media, and resulting colonies are counted after 2 to 3 days of incubation. In the basic clonogenic assay, cells are counted in a Thoma chamber at the microscope or by counting the cells directly on the plate. Alternatively, a cellular suspension can be deposited onto a 1-mm-thick pad of rich medium

with 2% agarose on a microscope slide, and after the incubation of slides for approximately 18 hours, the percentage of alive/dead cells is determined based on the ratio of plated cells that are able to form a micro-colony. As for mammalian cells, DNA fragmentation in yeast can be measured with the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, although before the attachment to slides and subsequent staining, yeast cells have to be fixed and the cell wall removed. Annexin V staining for the apoptosis-associated exposition of phosphatidyl serine also requires removal of the cell wall. Because the sensitive spheroplasts are easily damaged, integrity must be tested in parallel by a propidium iodine exclusion assay.

The level of intracellular reactive oxygen species (ROS) accumulation can be monitored by the incubation of cells with dihydrorhodamine 123 or dihydroethidium and by counting under a microscope or quantification in an appropriate fluorescent reader. Notably, these dyes are not able to detect specific ROS, and their signal is probably affected by metabolic rates.

Chromatin condensation and margination can be observed microscopically after DAPI staining or by using an electron microscope. Furthermore, fluorescence activated cell sorting (FACS) analysis is routinely used for ROS, annexin V, and propidium iodine staining and for the appearance of a sub-G0/G1 population.

2. PHYSIOLOGIC CONDITIONS THAT INDUCE APOPTOSIS IN YEAST

Among the physiologic conditions that can induce programmed cell death in yeast are (1) the presence of small quantities of the conjugation pheromone (mating-type pheromone), (2) the expansion of colonies on solid media, (3) the killer toxin, and (4) aging (Fabrizio et al., 2004b; Herker et al., 2004; Ivanovska and Hardwick, 2005; Longo et al., 1997; Reiter et al., 2005; Severin and Hyman, 2002; Vachova and Palkova, 2005).

2.1. Pheromone-induced cell death

Exposure of haploid yeast cells to small quantities of mating-type pheromones can induce apoptosis when a suitable mating partner is absent. If the mating occurs, apoptosis is prevented, suggesting that mating factor-induced cell death is used to eliminate infertile or otherwise damaged cells. Moreover, pheromone-induced apoptosis may favor the diploid state, which is likely to provide an adaptive advantage over the haploid state (Severin and Hyman, 2002). Cell death induced by a high concentration of mating pheromone is not apoptotic, but rather involves at least three different cellular pathways (Zhang et al., 2006). Under conditions such as scarce nutrition, diploid yeast cells can undergo meiosis and sporulation. Meiosis is important in that it increases genetic diversity and, as a consequence, fitness (Ahn et al., 2005). During meiosis, approximately 20% of cells undergo apoptosis, ensuring survival only for those cells that are better genetically adapted.

2.1.1. Colony growth

Yeast cells are able to use the nutrients released by their neighbors and can use this energy to reproduce, thus a theory of altruism seems feasible. In fact, although many of the data on yeast apoptosis have come from cells grown in liquid suspension, yeast in nature can grow as multicellular colonies that are capable of simple differentiation.

During the growth of colonies on solid media, apoptosis has been demonstrated to occur in a spatially restricted fashion, namely in the center of the colony. Cells present in this position are the oldest cells, and it has been demonstrated that their sacrifice improves viability of the younger cells located to the edge of the colony, consistent with the altruistic death program, described in the aging section that follows. Interestingly, the physical removal of the central region (dead zone)

from growing colonies caused a reduction in growth at the colony periphery.

2.1.2. Killer-induced cell death

The killer phenotype, a widespread phenomenon among yeasts, is typically associated with the secretion of a low-molecular-mass protein or glycoprotein toxin (killer toxin) that kills sensitive cells, without direct cell–cell contact in a two-step receptor-mediated process. In *S. cerevisiae*, three different killer toxins (K1, K2, and K28) have been identified so far, all encoded as precursors of the secreted alpha/beta toxins by cytoplasmic double-stranded RNA viruses. At low concentrations, all three virally encoded yeast toxins induce apoptotic cell death accompanied by apoptotic markers, whereas at high concentrations they induce nonapoptotic necrotic cell death. It can be concluded that when the toxin concentration is low, as in natural environments, killer yeast can eliminate sensitive competitor yeasts through the induction of apoptosis.

3. EXTERNAL STIMULI THAT INDUCE APOPTOSIS IN YEAST

Many environmental factors or drugs have been reported to be stimuli to commit cell death associated with typical hallmarks of metazoan apoptosis. Among the most studied inducers of apoptosis in yeast are the low dose of H₂O₂ and acetic acid, but also hyperosmotic stress, elevated temperature, amiodarone, osmotin, aspirin, HOCl, or merely sugar itself. More recently, it has been reported that the induction of programmed cell death (PCD) can also be achieved by metal ions, caffeine, sphingolipid, and dermaseptin, and this cell death can be meta caspase-dependent or -independent.

Yeasts may also be a powerful model for the screening or the development of cell death-directed drugs, overcoming the problem of cellular specificity in the design of antitumor drugs.

Paclitaxel, arsenic, bleomycin, and valproate represent the most well-studied antitumor drugs known to induce apoptosis in yeast, but the toxicity of doxorubicin, edelfosine, and other antitumor drugs has also been described (Almeida et al., 2008). The evidence indicates that the mechanisms of antitumor drug-induced apoptosis in yeast share some homology with those in mammalian cells and involve mitochondria, DNA fragmentation, and especially ROS production/accumulation.

Beside the studies of yeast apoptosis cells as a model to determine the cytotoxic effects of a multitude of drugs,

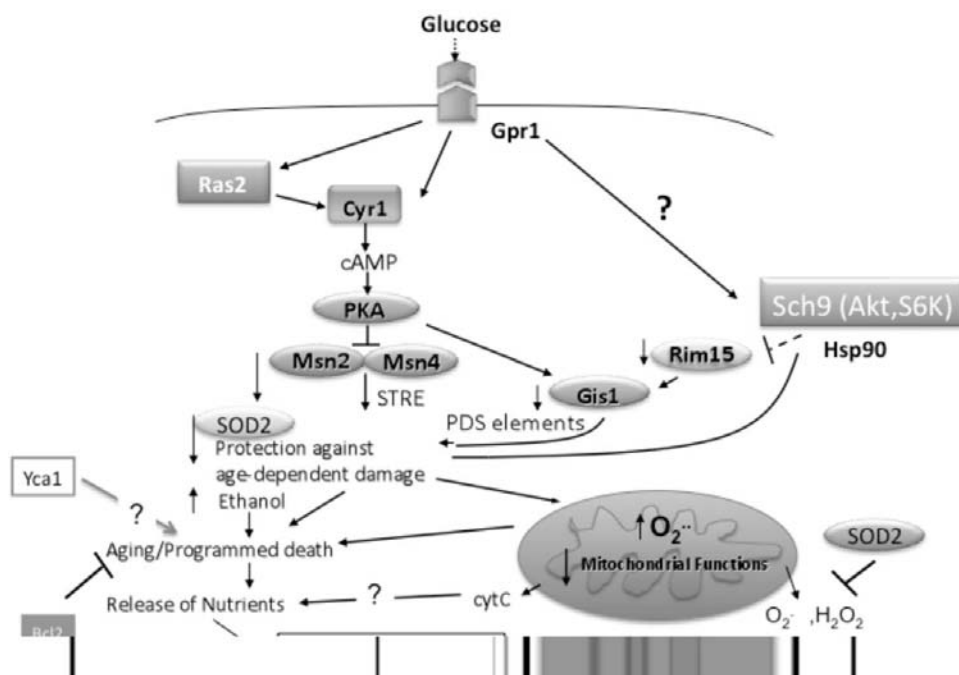


Figure 33-2. Aging and programmed cell death in yeast. The Ras and Sch9 pathways have been shown to regulate age-dependent programmed cell death in *S. cerevisiae* in part by controlling superoxide generation and stress resistance systems. Homologs of genes that affect PCD in yeast (SODs, cytochrome c, caspase) are also implicated in mammalian apoptosis.

they also could be directed to the design of new anti-fungal drugs. A new generation of antifungal drugs is required for many reasons, including the limitations associated with those currently in use and the increasing number of invasive fungal infections in immunocompromised patients. Thus the exploration of yeast PCD processes to identify molecules that trigger yeast cell death without causing serious side effects in human cells is extremely important.

4. THE GENETICS OF YEAST APOPTOSIS

Although a variety of toxins can promote apoptosis in *S. cerevisiae*, features of PCD were first described in yeast cells carrying the S565G substitution in the second ATPase domain (D2) of the *CDC48* gene, which codes for the AAA-ATPase and plays a role in cell division, ubiquitin-dependent endoplasmic reticulum-associated protein degradation (ERAD), and vesicle trafficking. Later on, it was observed that mutations in the VCP (valosin-containing protein), the metazoan homolog of the yeast *CDC48*, gave rise to apoptotic phenotypes in mammalian cell culture, in trypanosomes, *Drosophila*, and zebra fish. Moreover, mutations in VPC also trigger the human multisystem disorder called inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia (IBMPFD). More recently, other mutants in numerous cellular

fundamental processes, such as DNA replication, mitochondrial function, RNA, and protein stability, showed apoptotic phenotypes. These findings support the value of yeast as a model organism to study evolutionarily conserved mechanisms of apoptotic regulation.

5. PROGRAMMED AND ALTRUISTIC AGING

Much of the skepticism for the existence of apoptosis in yeast was supported by the apparent lack of an evolutionary base for the suicide of an organism. Perhaps one of the most convincing types of apoptosis in *S. cerevisiae* is that consistently observed during aging of yeast populations in a medium similar to that encountered in natural environments. Wild-type yeast aging chronologically in glucose/ethanol medium show features of apoptotic death such as generation of superoxide, nuclear condensation/fragmentation, and phosphatidylserine exposure; meta-caspase activation is also seen in some contents. ROS formation is enhanced in old cells, in agreement with a crucial role for superoxide in the mediation of yeast apoptosis during aging (see the remaining text in this section and Figure 33-2). Almeida et al. (2007) have recently provided evidence for the production of nitric oxide (NO) in aging yeast. According to their studies, NO contributes to superoxide production, and its removal by treatment with oxyhemoglobin, an

NO scavenger, extends the CLS (chronological life span) of wild-type cells. A number of genetic interventions that delay chronological aging and the appearance of the apoptotic features associated with it have been described. Among these are the disruption of the yeast meta-caspase *YCA1* gene and of *NDI1* (coding for the yeast homolog of the AIF-homologous mitochondrion associated inducer of death, AMID) and the over-expression of the stress-dependent transcription factor Yap1, although the most effective mutations known to block age-dependent apoptosis are the deletion of either *RAS2* or *SCH9* or both, which prolong the mean CLS by up to fivefold (Fabrizio et al., 2003; Fabrizio et al., 2001).

A nongenetic intervention that delays PCD and extends survival in wild-type yeast is calorie restriction/starvation obtained by switching yeast to water after growth in glucose medium (SDC [synthetic dextrose complete]). Starvation, as well as the reduction of the glucose concentration in the medium, can double the survival of *S. cerevisiae*, which suggests that yeast apoptosis depends on the type of environment encountered by the organism. Similar phenotypes including stress resistance and extended survival are shared between starved or ethanol-depleted cells and most of the long-lived mutants, including those lacking *SCH9* or *RAS2*. When aging wild-type cells incubated in glucose medium (SDC) are compared with long-lived *sch9* and *ras2* mutants, besides the apoptotic markers, major differences in stress response are observed. Both *sch9* and *ras2* mutants are less susceptible to oxidative stress than wild-type cells. Their resistance to oxidants depends in part on the expression of *SOD2*.

The presence of apoptotic markers and the role of nutrients and signal transduction pathways involved in nutrient signaling led to our hypothesis that aging *S. cerevisiae* activates a program that blocks cell protection and accelerates the death of the cells. The existence of such a program would be unlikely based on evolutionary theories, which rule out that a unicellular organism can activate a program that benefits other organisms. However, several lines of evidence have indicated that, for a population of millions or billions of yeast that have encountered an environment in which extracellular nutrients are scarce, cellular "suicide" represents a "group-level survival strategy." In fact, in approximately 50% of the aging wild-type cultures studied, cellular "regrowth" is observed (colony-forming units increase by up to 100 folds) after the great majority of the population has died. The percent of regrowth reaches more than 80% for cultures of mutants lacking *SOD1*, which codes for the cytosolic superoxide dismutase, suggesting a direct correlation between intracellular superoxide and frequency of regrowth. The regrowth phenotype, which

we called "adaptive regrowth," has been characterized extensively. Its characteristics are (1) correlation with frequency of nuclear mutations, which accumulate during aging, and (2) requirement for the nutrients released by the dead cells into the medium. Both features appear to be promoted by both cytoplasmic and mitochondrial superoxide, which, on the one hand, promotes accelerated cell death and consequently the release of nutrients and, on the other hand, causes DNA damage that facilitates the appearance of mutations with the ability to confer reentry into the cell cycle under conditions that normally prevent growth. In fact, the *sod1* deletion mutant is one of the mutants from the yeast knockout collection with the highest spontaneous mutation frequency. Importantly, the long-lived mutants, which are better protected against superoxide damage (including DNA damage), generally do not show adaptive regrowth, in agreement with a role for superoxide and protective systems in preventing the conditions necessary to achieve regrowth. Further analysis of yeast apoptosis during chronological aging has revealed that the pH of the medium affects cell survival. By day 3 the pH of the culture for cells grown in SDC medium of yeast cultures reaches 3 to 3.5. After adjustment to 6 to 7, the survival of the culture is significantly extended. In mammals the release of cytochrome c that triggers the caspase cascade activation in apoptotic cells is preceded by mitochondrial alkalinization and cytosol acidification. Cytochrome c release has also been observed in yeast treated with acetic acid, alpha factor, or over-expressing mammalian BAX, although its function in apoptosis activation has not been established yet. A possibility that needs to be investigated is whether the extracellular and consequently intracellular acidification observed in yeast aging cultures triggers apoptosis via the release of cytochrome c. In agreement with the role for a switch to water in doubling the survival, we determined that ethanol is an important promoter of age-dependent PCD in yeast. This carbon source is normally accumulated during fermentative growth but is not rapidly depleted in chronologically aging cultures of wild-type cells, at least in certain genetic backgrounds such as DBY746. When ethanol is removed from the incubation medium by evaporation, a significant life span extension is observed. Moreover, ethanol addition to cultures switched to water rapidly kills the yeast, suggesting an active role of ethanol in the activation of the death program in nondividing cultures, but also in the absence of all the nutrients required for growth. Recent work by Allen et al. (2006) has characterized yeast stationary phase cultures grown in rich medium by using an equilibrium density-gradient centrifugation method. After ~24 hours from the inoculation, they could

separate the cells in two fractions. The density of the lower fraction (high-density) increased steadily until day 7. The characterization of the two fractions revealed the following: (1) the high-density cells were mostly unbudded and showed several features of quiescent cells, whereas the low-density cells were both budded and unbudded and morphologically more similar to dividing cells (nonquiescent); (2) the low-density cells lost viability more rapidly than the others, were more sensitive to heat-shock, and produced more ROS than the quiescent cells; and (3) quiescent cells showed higher levels of apoptotic markers (DNA fragmentation and phosphatidylserine exposure) than non-quiescent cells.

It will be very important to continue to study the dynamics of the two types of cells to establish whether their ratio changes over time and whether quiescent cells become non-quiescent once the apoptotic program is activated. The apoptotic features described previously for the non-quiescent cells resemble those observed in wild-type cells aging chronologically in glucose/ethanol, suggesting the presence of non-quiescent cells in these cultures as well.

Adaptive regrowth therefore could represent a form of kin selection or group selection, with clear implication in relation to the aging theory. Thus an adaptive death program in the context of microbial populations is plausible and should be considered in parallel with classical evolutionary theories. Although natural selection primarily acts to increase the fitness of an individual, a multilevel selection process may more accurately reflect the complexity of the selection process, which must account for a population-based death program that appears to play a central role in the fitness of microbial populations. Notably, programmed aging/adaptive regrowth is only one of the strategies available to yeast populations to overcome periods of starvation. As mentioned earlier, incubation in water promotes life span extension and high levels of stress resistance, in agreement with the activation of a "survival program" that benefits all the individual yeast cells. Adaptive regrowth is observed also in budding yeast isolated from the natural environment, indicating that it is unlikely that the studies described above reflect only laboratory genotypes. Moreover, when laboratory strains are grown in grape extract (a medium that better reflects the natural environment for yeast), the survival curves and regrowth pattern are very similar to those obtained in glucose medium, strongly suggesting that adaptive regrowth is not an artifact owing to the use of synthetic medium.

Schizosaccharomyces pombe shares several similarities with *S. cerevisiae* in terms of chronological aging and its regulation. Although fewer studies have been

performed on PCD in *S. pombe*, apoptotic markers have been detected in cells expressing BAX/BAK and in mutants lacking the enzymes responsible for the biosynthesis of triacylglycerol. Furthermore, a homolog of the budding yeast meta-caspase Yca1 has been identified in the *S. pombe* genome. With respect to chronological aging, fission yeast show activation of meta-caspase activity, which is reduced in the long-lived *pka1* and *sck2* mutants (PKA and SCK2 are homologs of *S. cerevisiae* PKA and SCH9, respectively). Although still preliminary, these results and the conservation of the life regulatory pathways in the two yeast species suggests that an aging program and possibly adaptive regrowth also may be common among many yeast species.

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Brian L. Harry and Ding Xue

One point that emerges from the studies of programmed cell death in *C. elegans* and other organisms is the striking similarity of genes and gene pathways among organisms that are as superficially distinct as worms and humans.

– H. Robert Horvitz, 2002 Nobel Lecture

1. OVERVIEW

Since *Caenorhabditis elegans* was pioneered as a research tool in the early 1970s, it has become a window for peering into programmed cell death (PCD), also known as apoptosis. A soil-dwelling organism that is 1 mm in length and transparent, *C. elegans* is well suited for microscopic inquiry. Its reproductive cycle lasts 2 to 3 days and begins with either self-fertilization of a hermaphrodite or cross-fertilization between a hermaphrodite and a male. Forward genetic approaches using random mutagenesis and phenotypic screening, combined with a rapid generation turnover, have made *C. elegans* a powerful platform for discovery of new genes in PCD.

Cells in *C. elegans* can be tracked throughout development from their precursors because of a remarkably invariant cell lineage, which enables study of PCD at single-cell resolution. Of the 1,090 somatic cells that are generated during the life of a *C. elegans* hermaphrodite, 113 undergo PCD during embryogenesis and 18 during early larval development. These cell deaths are consistent and predictable according to their cell lineage. A third wave of PCD in the germline of adult worms occurs in a stochastic, lineage-independent manner. It has been estimated that roughly half of the 2,000 germ cells produced during a worm's lifespan undergo apoptosis. Apoptotic cells can be recognized by their highly refractile, raised disc morphology under differential interference contrast (DIC) microscopy (Figure 34-1). These unique features have made *C. elegans* an excellent model for elucidating fundamental aspects of apoptosis.

Apoptosis in multicellular eukaryotic systems can be cued by extrinsic signals that act through surface receptors or by intrinsic signals that trigger changes in mitochondria. Developmental PCD in *C. elegans* occurs

predominantly through the intrinsic pathway, also known as cell-autonomous death. Intrinsic signals initiate the specification phase of PCD, which decides the life versus death fate of a cell. Death specification pathways differ among cells, but they converge on a few targets, in particular, the cell death initiator *egl-1* (*egl*, egg-laying defective). In many cases, transcriptional upregulation of *egl-1* signifies the killing phase, which culminates in the activation of an aspartate-specific cysteine protease encoded by the *ced-3* gene (*ced*, cell death abnormal). The activated CED-3 protease then initiates the execution phase through cleavage and activation of downstream effectors that promote chromosomal degradation, mitochondrial elimination, alteration of the cell membrane, and removal of the dying cells. We discuss each of these phases for somatic PCD, beginning with the killing phase.

2. KILLING

A core genetic pathway is responsible for almost all PCD events during *C. elegans* development. Loss-of-function (*lf*) mutations in *egl-1*, *ced-4*, and *ced-3* result in survival of cells destined to die. Over-expression or increased function of these genes promotes ectopic cell deaths. On the other hand, *ced-9* (*lf*) mutations result in excessive cell death and lethality, whereas a gain-of-function (*gf*) mutation in *ced-9* prevents cell death. Epistasis analysis suggests that *egl-1* acts upstream of *ced-9* to induce apoptosis, *ced-9* acts upstream of *ced-4* to prevent apoptosis, and *ced-4* acts upstream of *ced-3* to induce apoptosis (Figure 34-2). When *egl-1* transcription is upregulated, the result of this pathway is activation of the CED-3 protease, which is a member of the aspartate-specific cysteine proteases, also known as caspases, that are involved in apoptosis in diverse species.

CED-3 cleaves its substrates at aspartate residues and is responsible for activating enzymes that degrade chromosomes, eliminate mitochondria, and promote cell surface changes to permit recognition by an engulfing cell (see Section 4, Execution).

Biochemical and structural experiments have corroborated the preceding genetic model and elucidated the functions of these proteins. In the early 1990s, CED-9 was found to be a homolog of the human antiapoptotic Bcl-2 (B-cell lymphoma 2) protein. This was a ground-breaking discovery because it provides the first example that a regulator of apoptosis is conserved between worms and humans. In living cells of *C. elegans*, CED-9 resides on the mitochondrial surface and tethers a CED-4 dimer, a homolog of human apoptotic protease activating factor 1 (Apaf1) that catalyzes caspase activation and apoptosis. Presumably, *egl-1* transcription is repressed in living cells, and CED-3 exists as an inactive proenzyme. When a cell death specification signal induces upregulation of *egl-1* transcription (see Section 3, Specification), the apoptotic cascade is initiated. EGL-1 is a Bcl-2 homology 3 (BH3) domain-only protein that binds and induces conformational change of CED-9, causing release of CED-4 dimers. CED-4 dimers subsequently form an oligomer, which catalyzes CED-3 proenzyme autoactivation. In a particular *ced-9(gf)* mutant, a glycine to glutamate substitution in the EGL-1-binding pocket of CED-9 impairs association of CED-9 with EGL-1 and EGL-1-induced CED-4 release, thereby blocking most PCD events during *C. elegans* development.

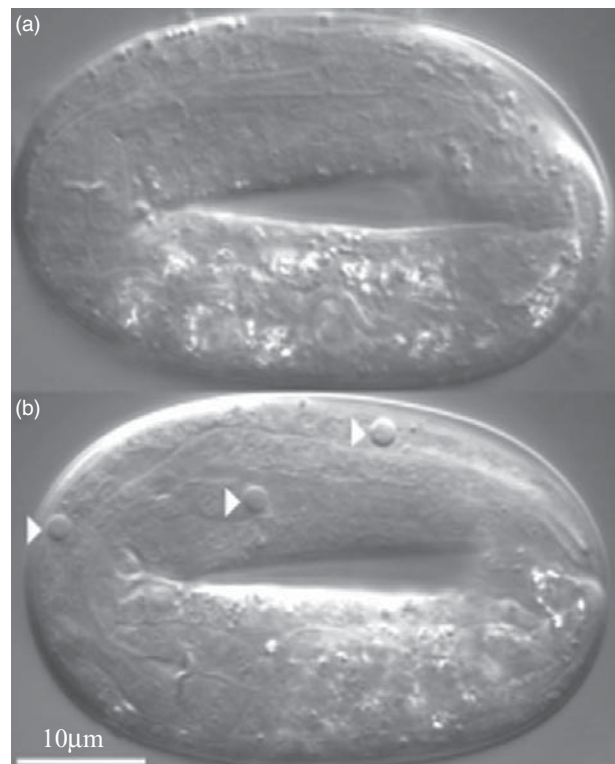


Figure 34-1. Apoptotic cells (arrowheads) can be recognized by their highly refractile, raised disc morphology under differential interference contrast (DIC) microscopy. A deficiency in engulfment of apoptotic cells caused by a *ced-1* loss-of-function mutation results in increased cell corpses in embryos (**B**) compared with wild-type embryos (**A**).

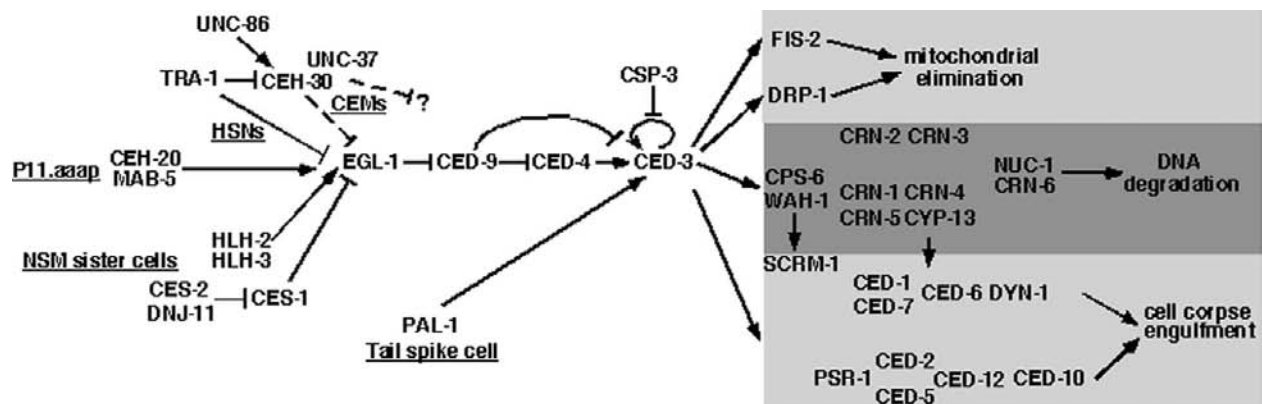


Figure 34-2. The genetic pathway of programmed cell death in *C. elegans*. *egl-1*, *ced-9*, *ced-4*, and *ced-3* constitute the central cell killing pathway, which culminates in the activation of the CED-3 caspase. The transcription of *egl-1* is regulated by a combination of transcriptional repressors and activators that determine life versus death fates of specific cells, such as male-specific chemosensory neurons (CEMs), hermaphrodite-specific neurons (HSNs), the P11.aap cell, and neurosecretory motor (NSM) sister cells. Other genes such as *ced-3* or *ceh-30* can also serve as checkpoints for cell death specification. The autoactivation of the CED-3 proenzyme is inhibited by CSP-3. Activated CED-3 caspase coordinates various cell death execution events by cleaving and activating downstream effectors, leading to mitochondrial elimination, DNA degradation and engulfment of apoptotic cells by phagocytes. Multiple pathways are found to mediate each of these cell death execution events.

The structure–function relationship of CED-9 is not identical to that of its mammalian counterpart. The C-terminal half of CED-9 resembles Bcl-2 and exerts its antiapoptotic activity by binding to CED-4 and preventing CED-4 from inducing CED-3 autoactivation. However, the N-terminus of CED-9, not found in Bcl-2, contains two CED-3 cleavage sites that are important for CED-9's death inhibitory activity, probably acting as a competitive substrate for CED-3. Surprisingly, a *ced-9(lf)* mutation, which causes ectopic cell death, and a weak *ced-3(lf)* mutation, which mildly inhibits cell death, in combination result in the survival of more cells that are destined to die in the pharynx of the worm than a weak *ced-3(lf)* mutation alone. This suggests that *ced-9* possesses a proapoptotic activity in addition to its antiapoptotic activity, though the reason for this is unclear. Currently, *egl-1* and *ced-9* are the only described Bcl-2 family members in *C. elegans*.

The primary function of CED-9 and Bcl-2 is to regulate the formation of the apoptosome, a structural scaffold of CED-4 in *C. elegans* and Apaf1 in mammals that activates caspase proenzymes. Though Bcl-2 does not directly bind Apaf1, it regulates the mitochondrial release of cytochrome c, a key component of the electron transport chain, which then promotes conformational change of monomeric Apaf1, binding and hydrolysis of ATP, and heptamerization into a wheel-shaped apoptosome. This results in exposure of caspase recruitment domains (CARD) in Apaf1 that engage and activate procaspase-9. Similarly, CED-4 is released from CED-9 as a dimer and forms an oligomeric apoptosome that activates the CED-3 proenzyme. This process does not require cytochrome c because CED-4 lacks the domain containing WD40 repeats that is important for Apaf1 binding to cytochrome c. Furthermore, CED-3 activation can be recapitulated in vitro using only recombinant CED-3, CED-4, CED-9, and EGL-1 in the absence of cytochrome c and adenosine triphosphate (ATP). Therefore, the involvement of cytochrome c and ATP hydrolysis in mammalian systems more tightly couples mitochondrial demise with apoptosis.

CED-3 is a member of the caspase family and likely acts by cleaving its substrates to initiate various cell death execution events. CED-3 is the only known caspase in *C. elegans* that has a definitive role in apoptosis, whereas multiple caspases have been implicated in apoptosis in mammalian systems. Given the essential role of *ced-3* in cell killing, regulating CED-3 proenzyme expression and activation is crucial for determining the fate of a cell. In *Drosophila* and mammalian systems, inhibitors of apoptosis (IAPs) negatively regulate procaspase activation and catalytic activity of

caspases. IAPs contain at least one baculoviral IAP repeat (BIR) domain that determines their caspase-binding specificity. In addition, other species contain IAP antagonists, such as Grim and Reaper in *Drosophila* and Smac/DIABLO in mammals. These factors bind IAPs and disrupt their activities. However, no IAP or IAP antagonist homologs have been identified in *C. elegans*. The first protein identified that negatively regulates the activity of CED-3 is CSP-3, which shares sequence similarity to the small subunit of CED-3. Biochemical analysis indicates that CSP-3 associates with the CED-3 proenzyme and inhibits its dimerization and autoactivation. However, CSP-3 does not block CED-3 activation induced by CED-4, nor does it inhibit the activated CED-3 protease. Therefore, it appears that *C. elegans* uses distinct mechanisms to regulate the activation of the CED-3 caspase and apoptosis.

Some apoptosis regulators do not function solely in apoptosis. For example, CED-4 participates in DNA damage–induced cell cycle arrest of germ cells and regulation of cell size and body size. In humans, caspase-1, -3, and -5 are important for cytokine maturation, demonstrating another apoptosis-independent function of these proteases. Currently, there is no demonstrated role for CED-3 outside of apoptosis. Although many questions remain, the conservation of several key cell death regulators and pathways between *C. elegans* and mammals is apparent.

3. SPECIFICATION

In *C. elegans*, cell death specification initiates the killing phase in a cell-autonomous manner, generally through upregulation of *egl-1* transcription. The *egl-1* gene consists of a short open reading frame flanked by a promoter and a sequence downstream of the coding region that contains several important *cis*-regulatory elements. A specific combination of activator(s) and repressor(s) is enlisted for regulation of *egl-1* transcription based on the lineage of a cell.

A unique program exists in hermaphrodite-specific neurons (HSNs) to regulate *egl-1* transcription in a sex-specific manner. HSNs innervate uterine and vulval muscles to expel fertilized eggs from the vulva of hermaphrodites. In males they are unnecessary and undergo apoptosis. A unique class of *egl-1(gf)* mutations causes ectopic HSN death in hermaphrodites by altering residues in a TRA-1A binding site located 6.4 kilobases downstream of the *egl-1* start codon. TRA-1A, a terminal sex-determination factor, is a Zinc finger transcription factor that promotes hermaphrodite development by repressing transcription of *egl-1* in hermaphrodite

HSNs and male-specific genes in general. Therefore, *egl-1(gf)* mutations cause derepression of *egl-1* expression and ectopic HSN death in hermaphrodites (Figure 34-2). Interestingly, *egl-1(gf)* animals do not display cell death defects in other cell types, indicating that this TRA-1A binding site must cooperate with other *cis*-elements to control appropriate *egl-1* expression in HSNs.

The complexity of *egl-1* transcriptional regulation is also apparent in determining the fates of neurosecretory motor (NSM) neurons, two serotonergic motor neurons, and their sister cells. During embryogenesis, a precursor neuroblast divides to generate the NSM and the NSM sister cell, the latter of which undergoes apoptosis. The fates of the NSM and NSM sister cell are determined by the expression level of CES-1 (*ces*, cell death specification), a Snail-like Zinc finger transcriptional repressor, as well as two helix-loop-helix (HLH) DNA-binding proteins HLH-2 and HLH-3, which induce *egl-1* transcription and are important for NSM sister cell death. Interestingly, the binding sites of CES-1 (Snail binding sites) and HLH-2/HLH-3 (E-box motifs) overlap, and in vitro CES-1 can compete with HLH-2/HLH-3 for binding to these *cis*-elements positioned 4 kilobases downstream of the *egl-1* start codon. When *ces-1* is activated in NSMs, HLH-2/HLH-3 binding to *egl-1* is excluded and the cells live. In NSM sister cells, *ces-1* is not expressed and the HLH-2/HLH-3 complex induces cell death. The expression of *ces-1* is in turn curbed by CES-2, a basic leucine zipper DNA-binding protein, and DNJ-11, a DnaJ domain-containing protein. Loss of *ces-2* or *dnj-11* results in transcriptional upregulation of *ces-1* and ectopic survival of the NSM sister cells (Figure 34-2). Comparison of mechanisms that regulate the deaths of NSM sister cells and sex-specific HSN neurons supports a model in which *egl-1* is intricately regulated by a combination of transcriptional activators and repressors.

The death of some of the ventral cord cells offers another glimpse into how *egl-1* transcription is regulated in specific cells. Ventral cord precursor cells give rise to a variety of cell types, including motor neurons and hypodermal cells. One of these cells, P11.aaap (the posterior daughter of the anterior daughter of the anterior daughter of the anterior daughter of the P11 ventral cord precursor cell) normally undergoes cell death. PCD of P11.aaap requires *ceh-20*, which encodes the *C. elegans* homolog of *Pbx* (pre-B-cell leukemia transcription factor), and the homeobox (Hox) gene *mab-5*. A complex of CEH-20 and MAB-5 has been shown to bind a *Pbx/Hox* site in the *egl-1* gene to activate *egl-1* transcription. Interestingly, *ceh-20* and *mab-5* are also expressed in P11.aaaa, the sister of P11.aaap that lives, indicating that an additional mechanism or a transcription

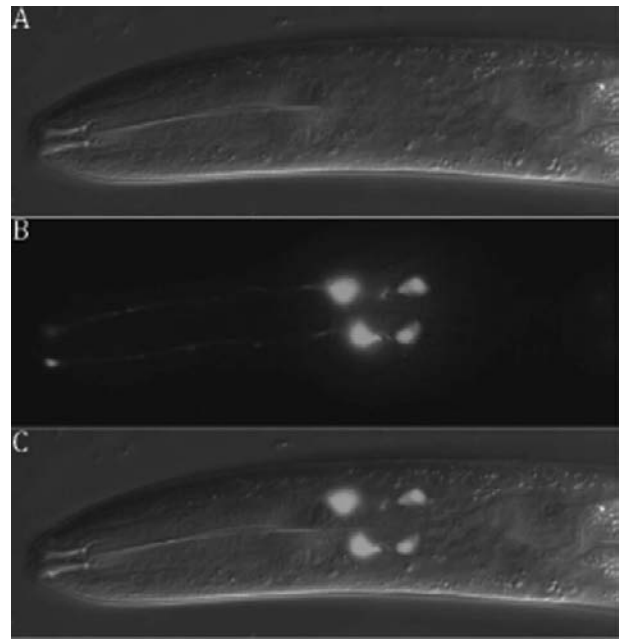


Figure 34-3. The four male-specific chemosensory (CEM) neurons located in the cephalic region of the animal undergo programmed cell death in hermaphrodites. The CEM neurons in males can be labeled by a *P_{pkd-2}gfp* reporter construct. DIC (A), GFP (B), and merged DIC/GFP (C) images of male CEM neurons are shown. See Color Plate 44.

repressor exists to prevent *egl-1* transcription in the P11.aaaa cell. Therefore, the P11.aaap cell, HSNs, and NSM sister cells use different sets of transcription factors but a similar paradigm to control *egl-1* expression and activation of apoptosis.

Although the current theme of cell death specification revolves around regulation of *egl-1* transcription, exceptions to this rule have been noted. *C. elegans* tail-spine cells produce a bundle of microtubule filaments over which the cuticle of the tail forms. They are unique in that they survive more than 5 hours before they die, much longer than other cells that die during development. *ced-3* and *ced-4* are absolutely required for tail-spine cell death, but *egl-1* is only partially required. Interestingly, tail-spine cell death depends on upregulation of *ced-3* by *pal-1* (Figure 34-2), which encodes a homolog of the Caudal homeodomain protein that can bind the *ced-3* promoter. However, PAL-1 is expressed in many other cells that do not undergo cell death, indicating that additional factors exist either to promote tail-spine cell death or to inhibit PAL-1-induced cell death in other cells.

The death specification of male-specific chemosensory neurons (CEMs, Figure 34-3) provides yet another example of how different developmental cues can converge at a non-*egl-1* checkpoint to control the life versus death fate of a cell. Surviving CEMs require the *ceh-30* gene, which encodes a BarH homeodomain protein that

acts with the transcriptional repressor UNC-37/Groucho to protect CEMs from undergoing apoptosis in males. Interestingly, the second intron of the *ceh-30* gene contains two adjacent *cis*-elements that are binding sites for TRA-1A, the terminal sex determination factor, and UNC-86, a POU-type homeodomain protein that specifies the CEM cell fates. In vitro TRA-1A interacts directly with UNC-86 on intron 2 and may suppress transcriptional activation of *ceh-30* by UNC-86, leading to activation of CEM cell death in hermaphrodites that have a high level of TRA-1A. Thus *ceh-30* integrates the sex determination signal TRA-1A and the cell fate determination and survival signal UNC-86 to control sex-specific death of CEMs (Figure 34-2). There is evidence that non-*egl-1* cell death activator(s) may be directly regulated by CEH-30/UNC-37, although the precise target of CEH-30/UNC-37-mediated transcriptional repression is unclear.

4. EXECUTION

Once the CED-3 caspase is activated, it orchestrates different cell death execution events by cleaving and activating multiple downstream effectors, leading to chromosomal fragmentation, mitochondrial elimination, and removal of apoptotic cells.

4.1. DNA degradation

Fragmentation of chromosomes is a hallmark of apoptosis that aids in dismantling the cell. In dying cells, chromosomes condense and are cleaved between nucleosomes, creating approximately 180 base pair fragments and permanently preventing DNA replication. The contribution of deoxyribonucleases to apoptosis has been enigmatic for many years. It was originally thought that DNA degradation was an expendable event late in apoptosis, because inactivation of the first identified apoptotic nuclease gene, *nuc-1* (*nuc*, abnormal nuclease), did not affect cell death activation or other aspects of apoptosis. Recent studies, however, have shown that DNA degradation actually facilitates apoptosis and clearance of a dying cell.

Currently, at least 10 genes are important for chromosomal degradation, several of which also facilitate clearance of the dying cell by a neighboring cell in *C. elegans*, which do not have professional phagocytes. These include *nuc-1*, *wah-1* (*wah*, worm AIF homolog), *cps-6* (*cps*, CED-3 protease suppressor), *cyp-13* (*cyp*, cyclophilins), and *crn-1* to *crn-6* (*crn*, cell death-related nuclease). Loss or reduction of activity in any of these genes results in the accumulation of cells with

3' hydroxyl DNA breaks in *C. elegans* embryos that can be labeled by the terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay. With the exception of *nuc-1* and *crn-6*, a defect in any of these genes also causes delayed or reduced cell death in sensitized genetic backgrounds. These genes seem to act in two distinct pathways to promote apoptotic DNA degradation, with *cps-6*, *wah-1*, *crn-1*, *crn-4*, *crn-5*, and *cyp-13* acting in one pathway and *crn-2* and *crn-3* acting in another. Interestingly, disrupting both DNA degradation pathways causes a defect in cell corpse engulfment at all stages of embryonic development. One possible explanation for this is that the DNA fragmentation process may facilitate the generation or exposure of "eat me" signals for phagocytosis. For example, nucleosomes can be presented on the surface of T cells and enhance phagocytosis by dendritic cells. However, the exact mechanism by which chromosomal fragmentation promotes clearance of apoptotic cells in *C. elegans* is unclear.

Both *cps-6* and *wah-1* encode mitochondrial proteins that are homologs of human endonuclease G (endoG) and apoptosis-inducing factor (AIF), respectively, which are also important for apoptotic DNA degradation in mammals. WAH-1 is released from the mitochondria by the cell death initiator EGL-1 and translocates to the nucleus in a CED-3-dependent manner. WAH-1 also physically binds and enhances the endonuclease activity of CPS-6. Moreover, CPS-6 may form a large DNA degradation complex with CRN-1, CRN-4, CRN-5, and CYP-13 (named the degradeosome) to promote stepwise DNA fragmentation, starting from generation of DNA nicks to single-stranded gaps to double-stranded breaks. On the other hand, *nuc-1* and *crn-6* encode type II acidic DNases that affect neither cell death nor engulfment. These two nucleases may act at a later stage of the cell death and DNA degradation process.

4.2. Mitochondrial elimination

As described previously, mitochondria play a central role in the killing phase of apoptosis in mammals. Mitochondria undergo dramatic morphological changes during apoptosis, including fragmentation, reorganization of cristae structures, and increased permeability of the outer mitochondrial membrane, all of which have been proposed to promote release of proapoptotic factors such as cytochrome c and Smac/Diablo from mitochondria of mammals. There are also reports that mitochondria are reduced or lost during apoptosis, which would eliminate cellular energy production and contribute to the demise of the cell. A comprehensive genetic and cell biological analysis of components of the *C. elegans*

mitochondria fission and fusion machinery, such as the dynamin GTPases DRP-1 (*drp*, dynamin-related protein), FZO-1 (*fzo*, Fzo mitochondrial fusion protein related), and EAT-3 (*eat*, eating defective), reveals that defects in mitochondria fission or fusion in *C. elegans* do not affect apoptosis activation. However, loss of DRP-1 and FIS-2, a homolog of the human Fis1 fission protein, does cause a mild cell death defect that can be detected in sensitized genetic backgrounds, suggesting that *fis-2* and *drp-1* have minor proapoptotic roles. Genetic epistatic analysis suggests that *fis-2* and *drp-1* act independently of one another and downstream of *ced-3* to promote apoptosis. Analysis by electron microscopy indicates that mitochondria normally reduced in size or eliminated in apoptotic cells persist in worms deficient in *fis-2* or *drp-1*, suggesting that DRP-1 and FIS-2 play a role in promoting mitochondrial elimination during apoptosis. Interestingly, active CED-3 protease can cleave DRP-1, and such cleavage is important for DRP-1's proapoptotic function, but not for its function in mitochondrial fission. Furthermore, the carboxyl terminal cleavage product of DRP-1 appears to be important for activating its proapoptotic function. Therefore, *fis-2* and *drp-1* represent two novel cell death execution pathways acting downstream of *ced-3* to promote mitochondrial elimination (Figure 34-2).

4.3. Engulfment

The timely removal of dying cells is important for development and tissue homeostasis in all organisms, in addition to immune responses and resolution of inflammation in mammals. Apoptotic cells that are not cleared undergo secondary necrosis, which could lead to tissue injury. Genetic analyses have identified many genes that are important for engulfment of apoptotic cells in *C. elegans*. These genes seem to work in two partially redundant pathways, with *ced-1*, *ced-6*, *ced-7*, and *dyn-1* (*dyn*, dynamin-related) acting in one pathway and *ced-2*, *ced-5*, *ced-10*, and *ced-12* working in another. Most of these genes are required in the engulfing cell, although the activity of *ced-7* is also needed in the dying cell. CED-1 is similar to the human scavenging receptor SREC and may function as a receptor on engulfing cells because it clusters around cell corpses in *C. elegans*. CED-1 clustering requires CED-7, an ATP-binding cassette (ABC) transporter, suggesting that CED-7 may play a role in transporting a signal that CED-1 recognizes or in mediating homophilic interaction between CED-7 on the dying cell and CED-7 on the engulfing cell. The ligands recognized by CED-1 have not been identified,

but CED-6 binds the intracellular portion of CED-1 via a phosphotyrosine binding (PTB) domain. CED-6 may transduce the engulfment signal to reorganize the engulfing cell membrane in response to CED-1 activation, probably through DYN-1, a *C. elegans* large dynamin GTPase. DYN-1 appears to mediate the internalization and degradation of dying cells by delivering intracellular vesicles to phagocytic cups to support pseudopod extension and to phagosomes to support their maturation and eventual digestion of apoptotic cells. After the internalization of apoptotic cells, multiple Rab GTPases and the HOPS complex mediate the maturation of phagosomes and the degradation of apoptotic cells.

In the other engulfment pathway, *ced-2*, *ced-5*, *ced-10*, and *ced-12* encode conserved components of the Rac GTPase signaling pathway that are important for rearrangement of the actin cytoskeleton in cell migration and engulfment. CED-2 is a CrkII-like adaptor with one SH2 (Src homology) and two SH3 domains that physically interacts with CED-5, the homolog of human DOCK180. CED-10, the *C. elegans* homolog of mammalian Rac GTPase, controls cytoskeletal dynamics and cell-shape changes downstream of CED-2 and CED-5. CED-12, a homolog of mammalian ELMO1, contains a potential PH (pleckstrin homology) domain and an SH3-binding motif through which it can interact with the CED-2/CED-5 complex. Genetic analyses indicate that *ced-2*, *ced-5*, and *ced-12* function at the same step but upstream of *ced-10* in the engulfment process. Biochemical analyses suggest that CED-2, CED-5, and CED-12 form a ternary complex to activate CED-10 GTPase. The phagocyte receptor for this pathway is poorly characterized. One candidate is PSR-1, the worm phosphatidylserine receptor homolog. PSR-1 binds specifically to cells exposing phosphatidylserine (PS) on their surface and acts in the CED-2/CED-5/CED-12 pathway to promote cell corpse engulfment, probably through interacting with CED-5 and CED-12. However, the engulfment defect of the *psr-1(lf)* mutant is significantly weaker than that of the *ced-2* or *ced-5* mutants, suggesting that other phagocyte receptors must also act in this pathway.

Almost all of the genes previously discussed act in phagocytes. Little is known, however, about the "eat-me" signals expressed by apoptotic cells. PS, which normally is restricted to the inner leaflet of the plasma membrane, is externalized during apoptosis and can serve as an "eat-me" signal to trigger phagocytosis. Recently, PS exposure on the surface of apoptotic cells in *C. elegans* has been demonstrated to be important for removal of apoptotic cells. Interestingly, the mitochondrial proapoptotic factor, WAH-1, is involved in promoting PS

externalization in apoptotic cells, in addition to its role in promoting apoptotic DNA degradation. WAH-1 appears to accomplish this by binding the phospholipid scramblase SCRM-1 and activating its bidirectional lipid scramblase activity, leading to the exposure of PS on the cell surface. Surface-exposed PS not only results in engulfment of apoptotic cells by phagocytes, but can also lead to engulfment of living cells that ectopically expose PS. This occurs in worms lacking the aminophospholipid translocase TAT-1 that maintains PS asymmetry. The engulfment of living cells by phagocytes in the *tat-1* mutant can be blocked by loss-of-function mutations in *psr-1* or *ced-1*, two putative engulfment receptors, suggesting that externalized PS can serve as “eat-me” signals for both engulfment pathways.

Phagocytosis not only removes cell corpses generated by PCD, but also actively promotes apoptosis. The mechanism by which this occurs is unknown and will be a topic of future study. The two engulfment pathways previously described are also important for the removal of necrotic cell corpses. The engulfment of apoptotic corpses is distinct from other forms of phagocytosis, such as antibody- or complement-mediated uptake of pathogens by mammalian immune cells, but this process seems highly conserved from *C. elegans* to humans.

Several other genes have also been implicated in cell death execution, including *ced-8*, which appears to control the timing of cell death and encodes a protein similar to human XK, a putative membrane transport protein.

5. SUMMARY

C. elegans has been a model organism for investigation of basic mechanisms of apoptosis. Its small size, transparent body, invariant cell lineage, and rapid reproductive rate make it particularly useful for experimental inquiry after genetic manipulation. Although the killing pathway comprising *egl-1*, *ced-9*, *ced-4*, and *ced-3* seems to be responsible for most somatic cell death events, regulation of *egl-1* transcription has been defined in only a handful of the 131 somatic cells that invariantly die during *C. elegans* development. In some cases, such as the tail-spike cell and CEMs, *egl-1* transcription does not seem to be a mandatory event for apoptosis. The mitochondrion appears to be a critical site for cell death regulation, as the CED-4/CED-9 complex localizes to mitochondria, and EGL-1 induces release of CED-4 from mitochondria and ultimately autoactivation of CED-3. Moreover, two mitochondrial factors, WAH-1 and CPS-6, are released from mitochondria during apoptosis to promote DNA degradation and PS externalization, two

important cell death execution events. The CED-3 caspase likely activates multiple cell death execution pathways, including two pathways that are responsible for cleaving internucleosomal DNA and two pathways for mitochondria elimination. Finally, engulfing cells use two partially redundant pathways to uptake and digest apoptotic cells.

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In animals, programmed cell death (PCD) is a universal feature of development and is critical for adaptive responses during cellular injury. In both vertebrates and invertebrates, dying cells often progress through a series of ultrastructural changes referred to as apoptosis. This form of PCD involves condensation of nuclear material and fragmentation into “apoptotic bodies” that are eventually engulfed by phagocytes. It is well established that apoptosis requires genetic functions within the dying cell and that the underlying molecular machinery is evolutionarily conserved. Studies in model systems have elaborated common control points in pathways that regulate cell death. However, when considered within larger networks of interactions, the importance of these regulatory “linchpins” can vary across different cell types and across different species. Here, we consider these similarities and differences and discuss how the *Drosophila* model organism may shed light on evolutionary pressures that fundamentally shaped this biological process.

1. CORE MOLECULES SPECIFYING APOPTOTIC CELL DEATH ARE CONSERVED

Seminal discoveries that illuminated core regulators of cell death were made in *Caenorhabditis elegans*. Prompted by the cloning of CED genes necessary for PCD in the nematode, our collective understanding of apoptotic cell death has grown at an astonishing pace. In this model system, precisely 131 of 1,090 cells undergo programmed cell death during development. Key molecules that specify worm PCD participate in precise interactions. These include the proapoptotic BH3-only Bcl-2 family homolog, *egl-1*, the antiapoptotic Bcl-2 family member, *ced-9*, the Apaf-1 ortholog, *ced-4*, and

the cysteine protease, *ced-3* (Figure 35-1). *ced-4* and *ced-3* are required for all cell deaths to occur, whereas, *ced-9* is required to prevent cell death. In worms, CED-9 physically binds to and inhibits CED-4, preventing activation of CED-3. The cell death machinery is activated when EGL-1 binds to CED-9 at the mitochondria, displacing bound CED-4, which is now free to bind to and activate CED-3. In this model, EGL-1 is a critical PCD determinant, and its presence or absence in any given cell is tightly controlled by transcriptional regulation.

Family members homologous to CED genes were found in diverse vertebrate models and in *Drosophila*, establishing that core components of the “apoptotic engine” are highly conserved. Figure 35-1 shows a prevailing model for the functional action of these genes and their counterparts in mammals and flies. In each organism, this genetic circuit controls activation of an apical caspase function via a multimeric complex referred to as the apoptosome. Caspases, in turn, mediate PCD by cleaving selected proteins, thereby reorganizing cellular physiology and promoting self-destruction. The fundamental importance of caspase action during apoptosis is underscored by the discovery of viral anti-apoptotic factors, such as p35 and inhibitors of apoptosis proteins (IAPs) that function as potent caspase inhibitors.

2. DROSOPHILA CASPASES AND PROXIMAL REGULATORS

Caspases are synthesized as dormant proenzymes, and during apoptosis, these precursors are processed to their active mature form by complex networks of proteolytic activation. Although caspase function is generally associated with apoptosis and immunity, recent discoveries from *Drosophila* and other models systems have

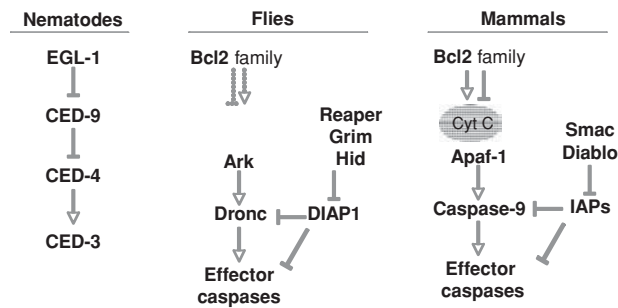


Figure 35-1. CED-3 represents the founding member of the proapoptotic cysteine proteases known as caspases, whereas the antiapoptotic gene, CED-9, shows structural and functional homology to the Bcl-2 family of proteins. A third gene, CED-4, bears homology to vertebrate APAF-1 and *Drosophila* Ark. These proteins activate apical caspase function via a multimeric complex referred to as the "apoptosome." Caspases in turn mediate PCD by cleaving selected proteins, thereby reorganizing cellular physiology and promoting self-destruction. An emerging picture for apoptosis control is consistent with a "gas and brake" model, whereby concurrent input from APAF-1/Dark adaptors, together with removal of IAP inhibition, drives caspase activation to levels that exceed a threshold necessary for apoptosis. From this viewpoint, a balance of opposing regulatory forces determines the status of apical caspase activity, but the relative contribution from positive regulators (APAF-1/Ced-4/Dark) and negative regulators (the IAP family) can vary among different cell types and species. For example, in the worm, mouse, and fly, mutations in positive regulators unanimously cause pronounced cell death defective phenotypes, but this is not consistently true with respect to the negative regulators. Therefore, although underlying components are broadly conserved, the regulatory linchpins in different cells and in different species can vary.

linked this family of enzymes to other functions, including cell type differentiation, sperm production, and migration. *Drosophila* is a premiere system for genetics, and in this genome, seven members of the caspase family are found. Flies have three initiator/apical caspases, Dronc, Dredd, and Strica (characterized by the presence of long N-terminal prodomains), and four effector caspases, Drice, Decay, Dcp-1, and Damm (characterized by short prodomains). Of the three apical caspases, Dronc is the primary apical caspase whose main function is in the apoptotic process. Dronc contains an N-terminal caspase recruitment domain (CARD), which binds to the CARD domain of the *Drosophila* Apaf-1 ortholog, Ark, to form a high-molecular-weight complex. Ark and Dronc, combined with dATP, form this multiprotein complex referred to as the apoptosome. On recruitment to the apoptosome, Dronc is processed and activated, which in turn perpetuates an amplified signal. This mode of recruitment and apoptosome formation is similar from worms to mammals, except that in mammals, cytochrome c is an essential component of this complex. Ark, the *Drosophila* Apaf-1 homolog,

contains WD40 repeats, a motif known for binding to cytochrome c in mammalian Apaf-1, but curiously, binding to cytochrome c is not required for formation of the fly apoptosome.

Evidence that Ark and Dronc are essential players in the *Drosophila* cell death pathway comes from genetic studies in which elimination of either gene results in almost complete inhibition of all PCD. Interestingly, however, when *ark* and *dronc* function are lost, not all cell deaths are abolished, and a few sporadic cell deaths are observed. Hence it seems likely that other components in the cell death pathway may compensate for the lack of a functional apoptosome, or perhaps redundant and/or parallel PCD pathways exist.

To date, known substrates of Dronc include the effector caspases Dcp-1 and Drice, both of which share homology with the mammalian effector caspase-3 and -7. Additionally, they are also sensitive to inhibition by the baculovirus caspase inhibitor, p35, which inhibits Dronc-dependent cell death, but not Dronc itself. *dcp-1* mutant animals are relatively healthy, exhibiting defects only in starvation-induced cell death during oogenesis. On the other hand, *drice* mutants exhibit marked reduced cell death in a variety of tissues, which produce semi-viable phenotypes. Interestingly, animals doubly mutated for *dcp-1* and *drice* produce cell death defective phenotypes that are far more severe than either single gene mutant alone, indicating that these two effector caspases may share partially redundant roles in *Drosophila* PCD.

3. IAPs PARTICIPATE IN CASPASE-DEPENDENT CELL DEATH

A second critical control point in fly PCD involves negative regulators of caspase activity known as IAPs. These proteins were discovered as potent baculoviral factors that function to suppress host cell death on infection. As a protein family, IAPs are defined by a 70-amino acid signature motif, known as the baculovirus IAP repeat (BIR), which mediates protein-protein interactions. Many members of this family also contain really interesting new gene (RING) finger domains associated with ubiquitin conjugation and E3 ligase activity. IAPs have received considerable attention because some members of this family physically bind – and directly inhibit – apoptotic caspases. The *Drosophila* genome encodes 3 IAPs: Diap-1, Diap-2, and dBruce. Diap-1 occupies a pivotal role in apoptosis pathways, and as a direct inhibitor, the gene and its protein product are the subject of intense study. In this model

system, loss of *diap-1* causes extensive cell death, and conversely, over-expression of *diap-1* blocks cell death. Interestingly, ectopic cell death caused by loss of *diap-1* is suppressed when levels of *ark*, *dronc*, or *drice* are decreased, highlighting its primary prosurvival function as a physiologic inhibitor of caspases. Diap-1 directly binds to Drice and Dcp-1 and physically prevents them from cleaving substrates by steric hindrance. Diap-1 also physically binds Dronc, but in this case, a distinct mechanism of negative regulation is used. Rather than steric hindrance or competitive inhibition, the E3 ligase activity of DIAP1 renders Dronc inactive by promoting ubiquitination and degradation of this apical caspase. Through its E3 ligase activity, Diap-1 also promotes ubiquitination and degradation of other proapoptotic proteins, such as Rpr, and also participates in autoubiquitination, thus targeting itself for proteasome-dependent degradation.

The discovery of BIR-containing genes in fly and vertebrate genomes, combined with discoveries that some IAPs can inhibit activated caspases through direct physical interactions, lends credibility to the idea that viruses acquired antiapoptotic molecules from their host genomes. This premise is strongly supported by genetic and biochemical evidence and is entirely consistent with the finding that DIAP1 is a pivotal apoptotic determinant in the fly. As a protein family, human IAPs are attractive therapeutic targets, and some preclinical findings validate applications for both antagonists and mimetics. In humans, as in flies, IAP-mediated inhibition of caspases involves direct physical contact between BIR domains and zymogenic forms of enzyme, and although the precise *in vivo* mechanism is not fully understood, it is already clear that ubiquitin-mediated protein degradation is important.

4. RPR PROTEINS ARE IAP ANTAGONISTS WITH CONSERVED BINDING PROPERTIES

Like many enzyme systems in any given cell, there exists a fine balance in the levels of processed caspases and IAPs. So how is this equilibrium tipped toward irreversible apoptotic cell death? The class of Reaper proteins, Rpr, Grim, Hid, and Skl (also known as RHG proteins), play important roles in tipping the balance in the direction of cell death. These proteins are potent apoptosis activators in *Drosophila*, and if heterologously expressed in vertebrate or human cell types, Reaper proteins are also sufficient to provoke acute apoptotic death in these systems as well. RHG proteins reside in the genomic interval known as the Reaper region, and when

deleted, nearly all apoptotic cell death is abolished. Reaper proteins are often expressed in patterns that anticipate PCD, and transcriptional regulation appears to be an important mechanism regulating the activity of these genes.

Once expressed, RHG proteins promote cell death through mechanisms that depend on binding to Diap-1. RPR proteins share an N-terminal motif referred to as the RHG motif or the IAP-binding motif (IBM). Residues within this motif can form direct contacts with the BIR domains of DIAP1, suggesting compelling models for how these proteins specify cell death. Although the precise mechanisms are not completely understood, RPR proteins and a fifth RHG containing protein, Jafrac2, are thought to antagonize DIAP1 through direct interactions that displace processed caspases (DRONC, DRICE, and DCP1) from negative regulation imposed by this IAP. In this way, derepression by RPR proteins, each with distinct affinities for a given BIR domain, promote liberation of apical and/or effector caspases from constitutive restraint by IAPs. Another way that RHG proteins regulate Diap-1 function involves ubiquitination (and autoubiquitination) activities, mediated by the RING domain in Diap1 that destabilize the protein. In mammals, orthologous activity is encoded by the Smac proteins, which similarly antagonize IAP function. Where examined, structural parallels and analogous contacts solved for the fly and mammalian complexes underscored common mechanisms of action for the fly and mammalian IAP antagonists.

Hence the balance of Diap-1, Dronc, and RHG proteins defines a critical control point in cell death regulation, where the family of RHG proteins play major roles by modulating DIAP1 stability and competing for binding partners. These protein-protein interactions specify apoptotic death in the sense that they determine whether caspase action surpasses a necessary threshold or exceeds a certain tipping point. Overall, the family of RPR proteins may be viewed as parallel switches that ultimately activate common caspase effectors. According to this model, apoptosis in development is specified by combinatorial expression patterns and selective constraints operating on these and perhaps other death activators.

5. *DROSOPHILA*: WHAT IS UPSTREAM OF THE APOPTOSOME?

In worms, the Bcl-2-mediated regulation of PCD occurs through direct physical interactions. Specifically,

Ced-9 physically binds and represses Ced-4 from activating Ced-3. However, in mammals, Bcl-2 proteins indirectly impact the apoptosome by regulating mitochondrial outer membrane permeability and specifying release of cytochrome c, a critical factor that promotes apoptosome assembly (see Figure 35-1). Thus, although Bcl-2 counterparts in worms and mammals occupy similar positions in the apoptosis pathway, they exert control over the apoptotic fate through distinct mechanisms. Furthermore, mammals have several members of both the anti- and proapoptotic members of Bcl-2 proteins. There also exists a family of proapoptotic Bcl-2 family members known as the BH3-only proteins. These proteins function to specify cell death by either inhibiting the antiapoptotic Bcl-2 proteins directly or by binding to and promoting the proapoptotic Bcl-2 family members. The antiapoptotic family of Bcl-2 proteins has been shown to play roles in preserving mitochondrial membrane integrity to prevent release of cytochrome c into the cytosol. The proapoptotic Bcl-2 proteins have been shown to promote cytochrome c release from the mitochondria, which initiates formation of the apoptosome complex by associating with WD40 repeat domains in monomeric Apaf-1 and inducing a conformational change in Apaf-1.

The *Drosophila* counterpart of Apaf-1, Ark, also contains WD40 repeats capable of binding to cytochrome c, and there is some evidence suggesting that cytochrome c may be altered during apoptosis. However, it is also clear that cytochrome c is not needed to form the apoptosome in vitro, and in vivo studies indicate that cytochrome c is not rate-limiting for apoptosome cell death. Hence the emerging data indicate little or no role for cytochrome c as a positive regulator of the apoptosome. Although genetic evidence supports this inference, a definitive resolution is complicated by the fact that two cytochrome c genes are encoded in the *Drosophila* genome. The fly genome also encodes two multidomain Bcl-2 family members, designated *debcl* and *buffy*. These share the canonical bcl-2 homology domains that define this protein family. On the basis of over-expression and gene silencing studies, *buffy* is considered an antiapoptotic gene, and *debcl* is thought to encode proapoptotic activity; recent genetic studies, however, suggest only limited roles for these in normal PCD.

6. CLOSING COMMENTS

Caspases are thought to be universal effectors of apoptotic cell death. These enzymes are synthesized as dormant “prozymogens,” and during apoptosis, they are

activated through complex cascades of proteolytic activation. By promoting the cleavage of selected substrates, these events ultimately reorganize cellular physiology and promote self-destruction. The network of regulatory molecules that control caspase action appears to be well conserved across species. In flies and mammals, an emerging picture for apoptosis control is consistent with a “gas and brake” model, whereby concurrent input from APAF-1/Dark adaptors, together with removal of IAP inhibition, drives caspase activation to levels that exceed a threshold necessary for apoptosis. From this viewpoint, the balance of opposing regulatory forces determines the status of apical caspase activity, but the relative contribution from positive regulators (APAF-1/Ced-4/Dark) and negative regulators (the IAP family) can vary among different cell types and species. For example, in the worm, mouse, and fly, mutations in positive regulators cause global cell death defective phenotypes, but this is not consistently true with respect to the negative regulators (IAPs). Therefore, although underlying components are broadly conserved, the regulatory linchpins in different cells and in different species can vary. Likewise, exit of cytochrome c from mitochondria appears to be a critical point of no return in mammalian cells but is unlikely to define the point of no return in either *Drosophila* or nematode systems. Consistent with this, elimination of the apoptosome or application of broad-spectrum caspase inhibitors preserves cell survival in both the fly and worm – but does not rescue mammalian cells from caspase-independent cell death. These differences among model systems can be exploited to illuminate important pathogenic states. For example, like fly cells in developing tissues, many human cancer cells also appear to rely heavily on IAP regulation as a means to prevent apoptogenic outcomes.

In closing, we note that prevailing models of apoptosis control are valuable, but as sometimes underscored by rigorous genetic analyses, they may also be inadequate in significant ways. For example, mice mutated for core pathway elements expose undefined alternate death pathways, independent of bax/bak, and likewise, both mouse and fly systems suggest that apoptogenic factors other than cytochrome c may impinge on the apoptosome. Other controversies focus on attempts to define the “point of no return,” which in principal represents a transition from a state in which cells could be rescued to a state in which protection is not possible. Here the *Drosophila* model may be particularly helpful because growing evidence for effector caspase activation without apoptosis suggests that pivotal

determinants downstream of this position need to be fully understood.

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1. INTRODUCTION

Although the fruit fly *Drosophila melanogaster* and the small nematode worm *Caenorhabditis elegans* have been crucial for advancing our understanding of the molecular mechanisms of cell death, there is a need for vertebrate model organisms to accelerate studies of human disease biology. A valuable vertebrate model system is the mouse *Mus musculus*, but this system suffers from the drawbacks of small litter sizes and in utero development. With these limitations in mind, zebrafish can provide unique advantages for in vivo experiments in a vertebrate model system to dissect conserved signal transduction pathways. In this chapter, we describe the zebrafish *Danio rerio* as a very useful vertebrate model system for studying cell death processes in both embryos and adults. First, we detail the advantages of the zebrafish animal model for discovering and analyzing cell death regulators. Second, we summarize a few studies in the field that have delineated distinct cell death mechanisms. Next, we highlight studies of developmental cell death and the very cancer-relevant p53 pathway in zebrafish, and we finish with some perspectives on the future of this exciting field of research. We hope to convey both a basic understanding of the strengths of this system and an overview of the important studies to date, which have enhanced our knowledge of the mechanisms that cells use to self-destruct when triggered by either developmental or environmental cues.

2. WHY USE ZEBRAFISH TO STUDY CELL DEATH?

Zebrafish offer a number of advantages for studying the cellular, molecular, and genetic processes of cell death. In this section, we highlight some of the major strengths of the zebrafish as a vertebrate model system for

studying cell death, and we also describe recent technologies that should have a profound impact on how researchers address the molecular genetics of cellular destruction in this model system.

2.1. The zebrafish life cycle

Zebrafish have a generation time similar to that of the mouse, reaching sexual maturity in 3 months (Figure 36-1). Unlike the mouse, however, embryonic development occurs ex utero, or outside the mother, and most developmental patterning is achieved within the first 24 hours after birth. One group of embryos (called a clutch) obtained from a single mating pair often contains hundreds of individuals that can be used to perform a variety of experiments, such as those detailed in the following pages. Zebrafish embryos are completely transparent, allowing easy visualization of such diverse developmental processes as muscle formation, brain patterning, heart tube folding, vasculogenesis, and blood flow. They develop pigment beginning at approximately 30 hours postfertilization (hpf), and they begin to freely swim at approximately 4 days postfertilization (dpf). Feeding also starts at 4 dpf, and the juvenile zebrafish grows rapidly from this stage until it reaches adulthood.

2.2. Molecular techniques to rapidly assess gene function in embryos

2.2.1. Studies of gene function using microinjections into early embryos

Both transient gain-of-function and loss-of-function experiments can be performed for any gene of interest by injections into the one-cell stage zebrafish embryo

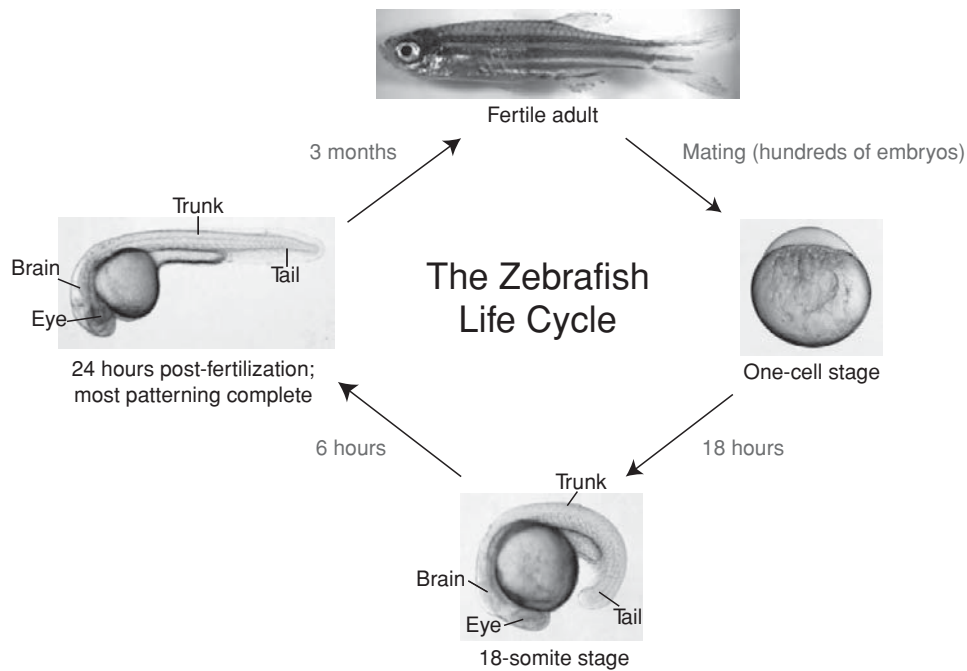


Figure 36-1. The zebrafish life cycle. Zebrafish adults can mate to produce hundreds of eggs in one clutch. On fertilization, the embryo is at the one-cell stage, when microinjection techniques can be employed to fill the entire developing animal with either morpholinos (for loss-of-function studies) or mRNA (for gain-of-function studies). After 18 hours, the embryo reaches the 18-somite stage, when tissues such as the muscle, brain, and eye are being formed. After 6 more hours, most embryonic patterning is complete, and the embryo has a clearly recognizable eye, brain, trunk, and tail. Shortly after this stage, the heart begins to beat and blood flow begins. After 3 months of development through larval and juvenile stages, the zebrafish becomes a fertile adult.

(0–45 minutes postfertilization). When injected at this stage of development, the material is distributed equally after each cell division into every cell of the developing animal. For *in vivo* gain-of-function experiments, mRNA is transcribed *in vitro* from a zebrafish expression vector, capped, and polyadenylated. Injection of this mRNA into one-cell stage embryos results in high levels of the gene product in every cell of the animal, and the effects can be monitored for approximately the first 24 hours of life. Conversely, transient loss-of-function experiments can be performed by injecting modified antisense oligonucleotides called morpholinos. These 25-mer oligos, which are commercially available, are capable of blocking either the translation or splicing of an mRNA, depending on the targeted site. Because of their chemical modification, morpholinos are very stable and can remain in the embryo for up to 3 to 5 days, continually knocking down levels of the targeted gene product.

2.2.2. In situ hybridization and immunohistochemistry

Two molecular techniques that can be powerfully used in zebrafish for studying localized gene expression and localized protein expression are *in situ* hybridization and immunohistochemistry, respectively. For at least the first

5 days of development, *in situ* hybridization using *in vitro* transcribed antisense riboprobes can be performed in whole animals to discover patterns of gene expression within developing tissues and to analyze the effects of gain- or loss-of-function experiments. Additionally over the same time period, immunohistochemistry can be performed to examine subcellular distribution or tissue localization of specific proteins. Immunohistochemistry and Western blotting are also accepted as the gold standards for assessing levels of protein knockdown in loss-of-function experiments.

2.3. Forward genetic screening

A major strength of the zebrafish over other vertebrate model systems, including the mouse, is that unbiased forward genetic screening can be performed in sensitized genetic backgrounds, even in moderately sized laboratories. The mutagen of choice used in the large-scale developmental screens of the mid-1990s is ethylnitrosourea (ENU). This alkylating agent causes random point mutations in the genomic DNA of treated animals, and incubating fertile male zebrafish in ENU causes mutagenesis of their developing spermatogonia. Crossing these mutagenized P0 males to wild-type females

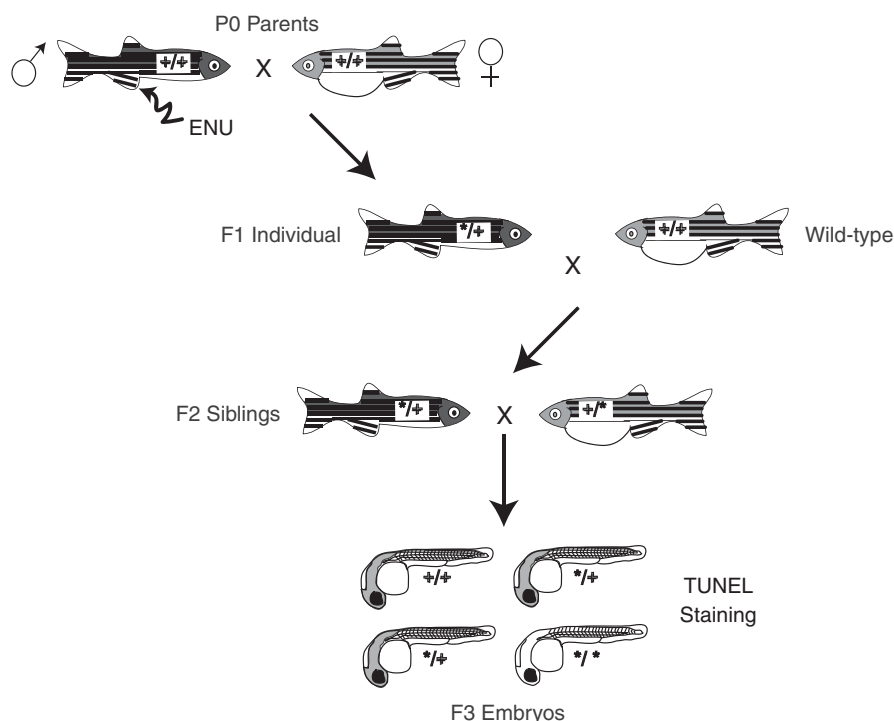


Figure 36-2. Recessive forward genetic screening in zebrafish. To initiate an unbiased forward genetic screen in zebrafish, adult males are treated with ethylnitrosourea (ENU), which induces point mutations randomly throughout the genome of developing spermatogonia. Subsequently, the mutagenized males are outcrossed to wild-type unmutagenized females to generate F1 individuals containing a variety of heterozygous mutations. These F1 individuals are then outcrossed to wild-type fish, and the resulting F2 families are kept separate from one another. Next, siblings from single F2 families are incrossed, and the F3 progeny are screened for recessive mutations that yield the phenotype(s) of interest. In this hypothetical screen, TUNEL staining is used after radiation-induced DNA damage to discover mutations that block DNA damage-induced apoptosis. Asterisks indicate mutant alleles, plusses indicate wild-type alleles, males are shown in black, and females are shown in gray.

and raising the clutches to adulthood yields F1 animals with individual sets of mutations, many of which will be gene-altering (as shown in Figure 36-2). Subsequently, F1 animals are outcrossed to wild-type fish, and for a standard F3 recessive screen, the F2 families are incrossed to yield embryos that harbor recessive mutations. Investigators can then analyze F3 clutches for their phenotype(s) of interest, knowing that the Mendelian percentage of 25 would be reflective of a true recessive mutation. After identifying a mutant based on an interesting phenotype, positional cloning techniques based on linkage analysis are used to map the mutation of interest and to clone the affected gene.

2.4. Drug and small-molecule screening

The small size, transparency, and permeability of the zebrafish embryo all combine to make this a perfect system for drug and small-molecule screening. Zebrafish embryos take up oxygen through diffusion, and by simply dissolving drugs or small molecules in the water of

a plate holding the fish, an investigator can introduce compounds into the animal's body. Zebrafish screens have already been successfully performed to discover a novel cell-cycle regulating compound, an angiogenesis regulator, a bone morphogenetic protein (Bmp) signaling inhibitor, and a novel compound that regulates hematopoietic stem-cell number. Because many embryos can be screened at one time in a multi-well format, the high-throughput nature of drug and small-molecule screening in zebrafish is comparable to that of cells growing in tissue culture.

2.5. Transgenesis

The ability to make transgenic animals is a major strength of the zebrafish system. Recently developed technologies using transposable elements as well as an *Isce-I* meganuclease strategy have greatly increased the efficiency of germline transgenesis to nearly 50%. By introducing exogenous genes into zebrafish embryos, researchers have already developed cancer models

in zebrafish for T-cell acute lymphoblastic leukemia, melanoma, pancreatic cancer, acute myeloid leukemia, and rhabdomyosarcoma. Other models such as one for neuroblastoma are being actively developed, and each of these models may be an excellent system for tumor-specific cell death-based drug discovery.

2.6. Targeted knockouts

A very recent technology that was developed in *Drosophila* and applied to zebrafish allows researchers to make targeted knockouts of their genes of interest. This strategy uses hybrid zinc-finger nucleases, which are modified enzymes that cleave DNA at specific 9–base-pair sequences. By injecting mRNA encoding two sets of three zinc-fingers coupled to heterodimeric cleavage domain variants of the endonuclease *FokI*, researchers can target a specific sequence within their gene of interest to excise DNA. The resulting non-homologous end-joining repair process frequently introduces microdeletions or frameshift mutations, resulting in early stop codons or missense mutations in the gene sequence. Furthermore, these loss-of-function lesions are highly penetrant and heritable, making it possible to create a stable knockout line for loss-of-function studies in both embryos and adults. Researchers can also create an allelic series for their gene of interest to study the varying effects of different types of mutations on gene function. This new technology should augment the utility of the zebrafish for reverse genetic approaches to inactivate genes and study the ensuing phenotypes. However, it should be noted that there is not yet a conditional knockout system in zebrafish as there is in mice, making the latter still advantageous for use in reverse genetic studies.

3. THE ZEBRAFISH MODEL ORGANISM IN CELL DEATH RESEARCH

The first systematic observations of altered cell death in zebrafish embryos came from large-scale mutagenesis screens of the mid-1990s in Tübingen, Germany, and Boston, MA. In these screens, scores of mutants with degenerating nervous system cells were isolated based on bright-field opacity of brain and spinal cord tissue, coupled with acridine orange staining to mark dead or dying cells. Since these early studies, researchers in the field have identified and functionally characterized factors important for intrinsic and extrinsic apoptosis, highlighting the large degree of conservation between zebrafish and mammalian apoptotic mechanisms. Additionally, a recent study in zebrafish revealed a new p53-independent apoptotic pathway that can induce

cell death after DNA damage in both zebrafish and human cells. Furthermore, although anoikis has not been formally studied in zebrafish, at least three studies have detailed either endothelial or keratinocyte apoptosis reminiscent of cells undergoing anoikis-induced cell death. Recent work has also uncovered autophagy in developing thymocytes after transgenic over-expression of both Myc and Bcl-2, the first example of this type of cell death mechanism in zebrafish. Necrotic models are infrequent in the zebrafish literature, but two studies indicate that apoptosis is not the sole mechanism of cellular destruction in the embryonic or adult fish in response to defects in pigment biogenesis or pathogen infection. This section highlights recent studies that demonstrate the relevance of the zebrafish system for discovering novel cell death regulators that are conserved in higher vertebrates.

3.1. Intrinsic apoptosis

Three studies have delineated and characterized the conservation of intrinsic apoptotic pathways in zebrafish. The first was a detailed genomic comparison performed by Inohara and Nunez in which they identified the majority of zebrafish apoptotic regulators by species comparison using genomic databases. In a second study, Kratz et al. (2006) used genomic comparisons and splice-site predictions to clone almost all of the zebrafish orthologs of mammalian apoptosis regulators (Table 36-1). They showed that zebrafish intrinsic apoptosis regulators have functions similar to those in mammals, with the p53-Puma axis dictating death after irradiation-induced DNA damage. Additionally, they showed that the zebrafish genome contains *bax1* and *bax2*, but they could not find a true *bak* ortholog. Through functional studies using mRNA over-expression, they showed that Bax2 functions like human Bak, because its apoptotic potential could only be blocked by Bcl-XL, Mcl1a, or Mcl1b, but not by Bcl-2. Meanwhile, all of the small BH3-only proapoptotic molecules functioned similar to those of their mammalian counterparts, confirming the conservation of intrinsic apoptotic machinery. Finally, in a recent article by Jette et al. (2008), biochemical data elegantly showed that zebrafish apoptotic regulators could interact with mammalian mitochondrial apoptotic proteins to potentially induce mitochondrial outer membrane permeabilization (MOMP) with generally similar kinetics to those of their mammalian counterparts. Additionally, this group cloned a true zebrafish *bim* ortholog, further highlighting the conservation of a wide range of BH3-only molecules in zebrafish. Consistent with

their *in vivo* effects, Bim and Puma acted most strongly to induce MOMP in the isolated mitochondria system used to assay the apoptotic potential of each molecule. Meanwhile, Bad seemed to act only as a sensitizer to irradiation-induced death, once again in agreement with the mammalian system. Interestingly, zebrafish BH3-only factors have a high degree of similarity to their mammalian counterparts solely in the pro-death BH3 domain, indicating that the other functions of these molecules may have diverged.

3.2. Extrinsic apoptosis

Two different studies have functionally described elements of the extrinsic apoptosis pathway in zebrafish. First, work from Kwan et al. (2006) showed that the zebrafish *death receptor* gene is a negative regulator of primitive hematopoiesis. Knockdown of the death receptor protein with morpholinos caused an increase in red blood cell number, indicating that extrinsic apoptotic pathways normally regulate cell numbers the erythrocyte population. A second study by Eimon and colleagues (2006) described cloning of the major components from the Fas ligand pathway, the Apo2L/Trail pathway, and the tumor necrosis factor (TNF) pathway. They found that the zebrafish genome contains one *fas ligand* and four *apo2L/trail* relatives, which they named death ligands (*dl*) 1a, 1b, 2, and 3. Additionally, zebrafish contain two different *tnf ligand* genes, which they named *ztnf1* and *ztnf2*. Genes encoding eight death domain (dd)-containing receptors were identified based on sequence homology and synteny to human orthologs, including a *tnr receptor* (*tnfr*), a *fas receptor* (*fas*), three *nerve growth factor receptors* (*ngfrs*), a *hematopoietic death receptor* (*hdr*; described in the Kwan et al. study), an *ovarian tnf receptor* (*otr*), and *death receptor 6* (*dr6*). Finally, genes encoding members of the death-inducing signaling complex associating with the activated receptors were identified. These included homolog of the *fas-associated death domain* (*fadd*), *trail-associated death domain* (*tradd*), and a number of initiator and effector caspases, including *caspase-8* and *caspase-10*.

To functionally characterize the activity of extrinsic apoptosis components, Eimon and colleagues (2006) over-expressed mRNA of the genes *hdr*, *otr*, *fas*, *dr6*, or *tnfr1* and found that injection of the first three mRNAs caused robust apoptosis by 8 hpf, as assayed by immunohistochemistry against activated caspase-3. By the same assay, other mRNAs that induced robust apoptosis between 8 hpf and 24 hpf included *fadd*, *tradd*, *caspase-2*, *caspase-3a*, *caspase-8a*, and *caspase-9*. Interestingly, over-expression of mRNA encoding the three death ligands (DL1a, 1b, and 2) caused apoptosis

Table 36-1. Analysis of cell death in zebrafish

Intrinsic Apoptosis Pathway Components	
Anti-apoptotic Bcl-2 proteins	Human homologs
zBcl-2-like protein 1 (zBclp1)	BCL-XL
zBcl-2-like protein 2 (zBclp2)	BCL-2
zMcl-1a	MCL-1
zMcl-1b	MCL-1
zNr13	BOO/DIVA
Pro-apoptotic BH3-only proteins	Human homologs
zBad	BAD
zBid	BID
zBik	BIK
zBmf1	BMF
zBmf2	BMF
zNoxa	NOXA
zPuma	PUMA
zBim	BIM
Pro-apoptotic multi-domain proteins	Human homologs
zBax1	BAX
zBax2	BAX
zBok1	BOK
zBok2	BOK
Extrinsic Apoptosis Pathway Components	
Extrinsic death ligands	Human homologs
zTnfl	LTa
zTnf2	LTa
zFasL	FASL
zDI1a	AP02L/TRAIL
zDI1b	AP02L/TRAIL
zDI2	AP02L/TRAIL
zDI3	AP02L/TRAIL
Extrinsic death receptors	Human homologs
zFas	FAS
zDr6	DR6
zNgfra	NGFR
zNgfrb	NGFR
zHdr	DR4/5
zOtr	DR4/5
zTnfi1	TNFR1
Note: List of cloned zebrafish intrinsic and extrinsic apoptotic factors, with human homologs listed on the right. Adapted primarily from Kratz et al. (2006) and Eimon et al. (2006), <i>Cell Death and Differentiation</i> 2006, Nature Publishing Group.	

specifically in cells of the notochord, the backbone of the zebrafish, at 24 hpf, as assayed by both caspase-3 activation and terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining. DL1b over-expression also caused death of erythrocytes, resulting in lower levels of O-dianisidine staining. These experiments highlight the power of the zebrafish embryo for assessing tissue-specific differences in apoptotic

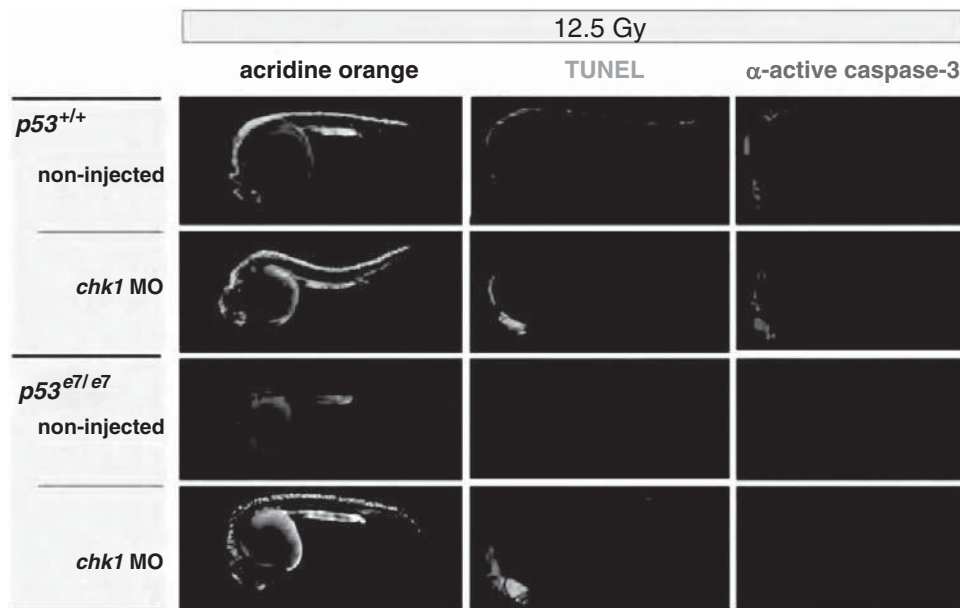


Figure 36-3. A rapid morpholino loss-of-function screen identifies *chk1* knockdown as a caspase-3-independent radiation sensitizer in *p53* mutant embryos. Fluorescent images of wild-type or *p53* mutant embryos at 25 hpf after irradiation (anterior to the left). TUNEL reactivity (middle column) after 12.5 Gy of irradiation recapitulates live AO labeling (left column). Embryos from the same experiment were immunostained with an anti-activated-caspase-3 antibody (right column). Note the absence of activated caspase-3 immunoreactivity in the irradiated *p53*^{e7/e7}*chk1*MO embryo. Reprinted from Sidi S, Sanda T, Kennedy RD, Hagen AT, Jette CA, Hoffmans R, Pascual J, Imamura S, Kishi S, Amatruda JF, Kanki JP, Green DR, D'Andrea AA, Look AT. Chk1 suppresses a caspase-2 apoptotic response to DNA damage that bypasses p53, Bcl-2, and caspase-3. *Cell*. 2008 May 30;133(5):864–77, with permission from Elsevier. See Color Plate 45.

potential downstream of various stimuli. Remarkably, loss-of-function experiments in zebrafish embryos using morpholinos targeted against *hdr*, *otr*, or *fadd* did not cause significant developmental defects, indicating that the extrinsic apoptotic pathway is not critical for early zebrafish development. This is similar to blockade of the intrinsic apoptotic pathway by injecting mRNA encoding Bcl-XL or Bcl-2, which has no obvious effect on embryogenesis. Thus apoptotic pathways in zebrafish embryos may be necessary only to kill cells after severe stress or to fine-tune the numbers of cells in a specific lineage.

3.3. Chk-1 suppressed apoptosis

In a morpholino screen to discover factors for which knockdown would restore DNA-damage induced apoptosis to *p53* mutant zebrafish, Sidi and colleagues (2008) elucidated a novel caspase-2 dependent apoptotic mechanism functioning downstream of Chk-1 inhibition that bypasses p53 and key components of both the intrinsic and extrinsic apoptotic pathways. This study highlighted the advantage of rapid analysis of loss of function for a panel of genes in the zebrafish embryo using morpholinos. Checkpoint kinases were quickly and systematically knocked down in a small-scale screen for modifiers of absent cell death in *p53*

mutant embryos. Furthermore, this group was able to examine epistatic interactions with Chk-1 inhibition (Figure 36-3) through Bcl-2 over-expression and assays for both Caspase-3 activation downstream (of which there was none) and caspase-2 activation (which became apparent only after Chk-1 knockdown). The analyses were extended into human cell culture, where Chk-1 shRNA studies in five independent *p53*-mutant or null cell lines confirmed the relevance of the new apoptotic pathway to human cancer cells. Because at least half of all human tumors are mutant for p53, Chk-1 inactivation represents a novel and potentially powerful approach to sensitize *p53*-mutant tumor cells to the apoptotic effects of DNA-damage inducing agents, such as radiotherapy. This study bolsters use of the zebrafish system for rapid analysis of cell death pathways that can subsequently be manipulated in human tumor cells.

3.4. Anoikis

Documentation of anoikis in zebrafish comes from work by three different laboratories showing that both endothelial cells and skin keratinocytes undergo apoptosis after abnormal detachment from their extracellular matrix. The first study showed that integrin-linked kinase (Ilk) loss-of-function in zebrafish embryos causes

a loss of endothelial cells that mimics the mouse knockout phenotype. Loss of integrin signaling from attached cells is a primary cause of anoikis, also called detachment-induced cell death. In a mouse cell culture model, this group identified a loss of integrin-matrix attachment in endothelial cells as a direct cause of apoptosis. Extending these studies to transgenic zebrafish with Egfp-labeled vascular endothelial cells, they observed that *Ilk*-specific morpholino injection caused a reduction in the number of endothelial cells in the trunk of the embryo. Although anoikis was not specifically discussed as the cause of apoptosis in this model, this study raises the intriguing possibility that matrix attachments are crucial for cellular survival in the developing zebrafish embryo.

It was recently shown that the zebrafish Birc2 protein also acts during development to promote endothelial cell integrity and survival. As a cellular inhibitor of apoptosis (clap), Birc2 associates with Tnfr signaling components to prevent downstream activation of extrinsic apoptosis pathways through caspase-8. Nuclear Factor kappa B (NF- κ B) signaling was known to be important for vascular integrity in other contexts, and this group established that zebrafish Birc2 likely functions through NF- κ B by rescuing *birc2* mutant fish with the NF- κ B-activating protein NEMO. Taken together, these results indicate that Birc2 may be a prosurvival factor acting downstream of external cues, such as integrin signaling, to maintain vascular integrity and endothelial cell survival.

Anoikis mechanisms also seem to be active in keratinocytes, the primary cell type found in the epidermis. Nowak and colleagues (2005) showed that the p53 signaling factor *Perp* functions as an antiapoptotic factor to maintain survival in cells undergoing transient developmental detachment from the underlying basement membrane. *Perp* morphants displayed extensive apoptosis in this tissue layer, and it is quite possible that the loss of integrin signaling during detachment triggered anoikis in this system. Future studies such as high-resolution confocal time-lapse microscopy with *ilk* morphants, *birc2* mutants, and *perp* morphants can further address whether anoikis-like mechanisms are truly acting to kill cells in the contexts of endothelial cell and keratinocyte detachment.

3.5. Autophagy

Recent work from Feng and colleagues has described the first evidence of autophagy in zebrafish. While analyzing adult fish with thymocytes over-expressing the oncogenes Myc and Bcl-2, these researchers dis-

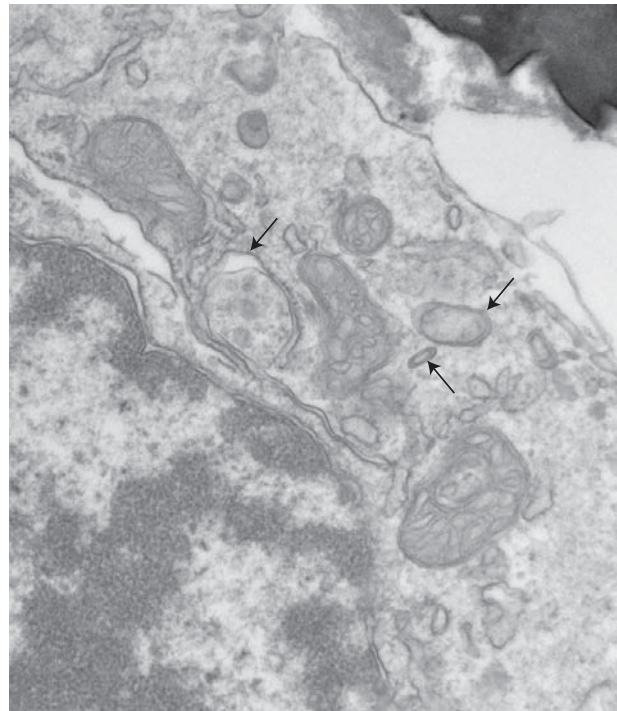


Figure 36-4. Autophagy in zebrafish. Electron micrograph of one representative thymocyte in a *rag2:egfp-bcl-2/rag2:myc* transgenic adult zebra fish. Note the double-membraned structures denoted by arrows. These structures contain mitochondria and cytoplasm, a hallmark of autophagy. Image courtesy of Dr. Hui Feng.

covered a “small cell” phenotype. On transmission electron microscopy (TEM) analysis, they found that these cells had obvious double-membraned structures, which are rarely observed in normal cells (Figure 36-4). These double-membraned structures contained cytosol or organelles such as mitochondria, typical features of autophagosomes. Furthermore, the autophagy marker LC-3 was clearly cleaved in the Myc/Bcl-2 thymocytes, another indication of autophagosome formation. These studies establish the existence of autophagic mechanisms in zebrafish placed under specific oncogenic stresses. However, the autophagy in this context seems to be serving a prosurvival role rather than a pro-death role, which is an emerging theme in tumor cell biology. Future studies are needed to determine whether autophagy plays any role during normal zebrafish development in either cell survival or cell death.

3.6. Necrosis

Necrotic cell death in zebrafish embryos can be distinguished from apoptosis by determining that the dying cells are TUNEL-negative, cannot be rescued by a pan-Caspase inhibitor, and display membrane rupture in electron micrographs. McNeill and colleagues

used these criteria to show that zebrafish with mutations in the *transient receptor potential melastatin 7* (*trpm7*) locus display necrosis in pigment-forming cells called melanophores. This necrosis is dependent on melanin synthesis, as inhibition of the melanin synthesis pathway blocked cell death in mutant embryos. Interestingly, *trpm7* is expressed in metastatic melanoma lines, indicating that the role of this gene as a prosurvival factor in melanin-producing cells may potentiate metastasis. Further studies of necrosis have been performed in models of tuberculosis infection, in which disease progression includes the formation of necrotic granulomas. Swaim and colleagues showed that infected cells display cellular breakdown characteristic of necrosis, although this group did not perform molecular analyses to definitively rule out any role of apoptotic mechanisms in cellular elimination.

4. DEVELOPMENTAL CELL DEATH IN ZEBRAFISH EMBRYOS

The majority of the cell death studies described in the preceding section pertain to gain- or loss-of-function experiments that examined the effects of changing the balance of cell death factors versus survival factors in zebrafish embryos or adult fish. However, it is also clear that developmental cell death occurs in zebrafish in a regulated fashion, ultimately creating the proper number of cells in each tissue of the body (Figure 36-5). In a study published in 2001, Cole and Ross used TUNEL staining over a series of developmental stages to create a map of cells undergoing apoptosis in the developing zebrafish embryo. From this and previous work, it was established that developmental cell death in zebrafish occurs primarily in nervous system tissues, including the transient Rohon-Beard sensory neurons, all of which die by caspase-9-dependent intrinsic programmed cell death between 18 hpf and 72 hpf. The death of this early sensory neuron population is mediated by a combination of neurotrophin signaling and electrical activity. Intriguingly, elegant studies have shown that the sensory arbors from these neurons persist long after the main cell bodies have died, providing a unique insight into the delayed mechanisms of the complete clearance of dead cells in the nervous system. Other tissues undergoing developmental apoptosis over a series of developmental stages include the eye, the nose (olfactory placode), the brain, the urogenital system, the germ cells, and the tail.

One of the major strengths of studying the zebrafish embryo is the ability to image cell biology in real-time using fluorescent reporter proteins or vital dyes. In a recent study from Peri and Nusslein-Volhard (2008),

Regions of developmental cell death

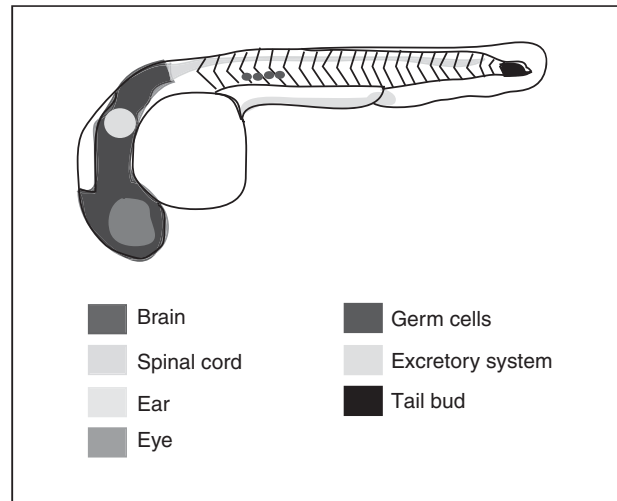


Figure 36-5. Cell death zones in developing zebrafish embryos. Regions of developmental cell death summarized in a 24-hpf embryo (anterior to the left). Note the diverse range of tissues that die during embryogenesis. All embryos are shown with anterior to the left. Reprinted with permission of Elsevier. See Color Plate 46.

the imaging of neuronal cell death and engulfment by microglia in the brain was achieved with unprecedented clarity. To prevent the accumulation of damaging cell-death products in surrounding cells, microglia (the phagocytes of the brain) quickly digest dying neurons by phagocytosis. Using a double-transgenic zebrafish line with membrane Egfp-labeled microglia and neuron-labeled DsRed, the researchers filmed the process of cellular death in the brain followed by the recognition, engulfment, and phagocytosis of apoptotic cells by neighboring microglia (Figure 36-6). They observed that phagocytic cups in the extended processes of microglia engulf dying neurons and transport them to the microglial cell body to eliminate them. Further work showed that the microglial protein Atp6v0a1 is critical for the digestion of dead neuronal cells within microglia through the formation of phagolysosomes. This exciting study further confirms that the imaging of cell death in zebrafish is a unique and powerful approach to screen for morpholinos, drugs, or mutations that affect fundamental apoptotic processes, such as cellular recognition and digestion after apoptosis.

5. THE P53 PATHWAY

Any discussion of cell death studies would be incomplete without mentioning the p53 pathway and its importance for apoptotic processes during development and in cancer. p53 is activated after cellular stress or DNA damage, and one of its major roles is as a

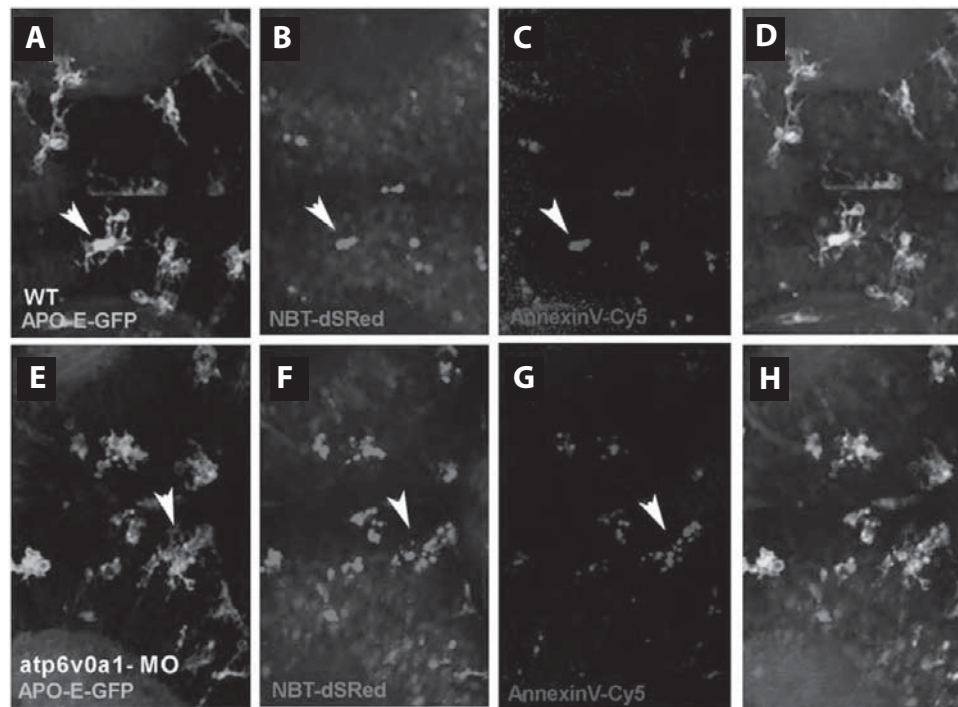


Figure 36-6. Images of microglia consuming dying neurons by phagocytosis in wild-type and *atpv0a1* morphant larvae. **(A–D)** Dorsal view of a wild-type fish head at 3 dpf (days postfertilization). Foci of apoptotic neurons are found inside wild-type microglia. **(A)** Apo-E-GFP expression. **(B)** NBT-dSRed expression. **(C)** AnnexinV-Cy5 labeling of apoptotic clusters. **(D)** Merge. White arrowhead points at one single apoptotic neuronal cluster. **(E–H)** Dorsal view of the fish head at 3 dpf in *atpv0a1* morpholino-injected embryos. Foci of apoptotic neurons are larger than in wild-type. **(E)** Apo-E-GFP expression. **(F)** NBTdSRed expression. **(G)** AnnexinV-Cy5 labeling of apoptotic clusters. **(H)** Merge. White arrowhead points at one single apoptotic neuronal cluster. The size of the cluster is larger when compared with that of the wild-type. Reprinted from Peri F, Nüsslein-Volhard C. Live imaging of neuronal degradation by microglia reveals a role for v0-ATPase a1 in phagosomal fusion in vivo. *Cell*. 2008 May 30;133(5):916–27, with permission from Elsevier. See Color Plate 47.

proapoptotic transcription factor. In zebrafish, a reverse genetics approach called TILLING (targeting-induced local lesions in genomes) was used to isolate a zebrafish *p53* mutant that is highly relevant to human cancer. As with most human *p53* mutations in cancer, the zebrafish mutant has a single missense mutation in the DNA-binding domain of *p53* that renders it incapable of activating transcription. Berghmans and colleagues (2005) showed that this mutant zebrafish line develops malignant peripheral nerve sheath tumors in nearly 30% of animals by 16.5 months of age. Early embryonic assays showed that the animals have a complete loss of DNA damage-induced apoptosis compared with controls. These studies laid the groundwork for the discovery of the Chk1-suppressed apoptotic pathway described earlier, and additional screens using unbiased mutagenesis approaches should further aid our understanding of pathways that can bypass the *p53* axis and promote cell death after DNA damage and other stresses.

In addition to DNA damage-based *p53* activation, recent work from Robu and colleagues described off-target activation of the *p53* pathway after the injection of

morpholinos designed to knock down gene function in zebrafish. A common toxic effect after injection of some morpholinos is extensive cell death in the brain and nervous system of injected embryos. This unintended side effect has confounded the analysis of apoptotic mechanisms, because investigators had been unable to disentangle the effects of gene knockdown from the effects of morpholino toxicity. However, work from this group showed that co-injection of a *p53* morpholino along with a morpholino targeted against the gene of interest can block the unintended nonspecific morpholino-induced cell death in the brain and nervous system. Thus, researchers can distinguish which tissue patterning and/or cell death mechanisms are likely to be a consequence of morpholino toxicity, and focus on the effects of specific knockdown of the protein of interest. Using a series of morpholinos that had been previously described to cause nervous system cell death, Robu and colleagues showed that simultaneously knocking down *p53* rescued the cell death defects without changing tissue-patterning defects that resulted from specific protein knockdown. This work created a new paradigm for

morpholino loss-of-function studies, and it also hinted at a new mechanism for p53 activation by injection of foreign nucleic acids. Though the molecular mechanism(s) for this activation are still unclear, the work suggests that cells have an internal p53-dependent sensor for detecting the presence of morpholino oligonucleotide stress and eliminating cells as a result. Of course, if researchers are studying an antiapoptotic molecule whose *specific* knockdown actually does trigger p53 activation, other types of controls such as phenotypic rescue or development of a stable genetic mutant become critical for establishing the validity of the observed effects.

6. PERSPECTIVES AND FUTURE DIRECTIONS

In this chapter, we have reviewed studies demonstrating that the zebrafish is a very useful model system for studying cell death processes that are relevant to human development and disease. Although the zebrafish model has been primarily used for studies of vertebrate developmental processes, this system is increasingly being used for studies of disease mechanisms. Technologies such as those employed in the articles we have highlighted are allowing researchers to quickly and effectively attack pressing questions in the field of cell death, and future approaches should further enhance the usefulness of this model system for rapidly assessing drug efficacy and the essential functions of cell death regulators. Zebrafish thus combine the strengths of flies and worms in genetic analysis based on phenotype assessment with the strengths of the mammalian mouse model in its relevance to human disease processes. From a muddy freshwater river in India, the zebrafish has begun to show its stripes as a premier animal model for studying vertebrate cell death pathways.

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