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Apoptosis and Cell Death: Relevance to Lung

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Introduction

In multicellular organisms, cell death plays an important role in development, morphogenesis, control of cell numbers, and removal of infected, mutated, or damaged cells. The term *apoptosis* was first coined in 1972 by Kerr et al.[1] to describe the morphologic features of a type of cell death that is distinct from necrosis and is today considered to represent programmed cell death. In fact, the evidence that a genetic program existed for physiologic cell death came from the developmental studies of the nematode *Caenorhabditis elegans*.[2] As time has progressed, however, apoptotic cell death has been shown to occur in many cell types under a variety of physiologic and pathologic conditions. Cells dying by apoptosis exhibit several characteristic morphologic features that include cell shrinkage, nuclear condensation, membrane blebbing, nuclear and cellular fragmentation into membrane-bound apoptotic bodies, and eventual phagocytosis of the fragmented cell (Figure 4.1).

Cell death is central to the normal development of multicellular organisms during embryogenesis and maintenance of tissue homeostasis in adults.[3] During development, sculpting of body parts is achieved through selective cell death, which imparts appropriate shape and creates required cavities in particular organs. In adults, cell death balances cell division as a homeostatic mechanism regulating constancy of tissue mass. Deletion of injured cells because of disease, genetic defects, aging, or exposure to toxins is also achieved by apoptosis. In essence, apoptotic cell death has important biologic roles not only in development and homeostasis but also in the pathogenesis of several disease processes.

Dysregulation of apoptosis is found in a wide spectrum of human diseases, including cancer, autoimmune diseases, neurodegenerative diseases, ischemic diseases, viral infections,[4] and lung diseases.[5] Our knowledge of cell death and the mechanisms of its regulation increased dramatically in the past two decades with the discovery of death genes in *C. elegans*[2] and their counterparts in mammals.

Apoptosis and Other Forms of Cell Death

Earlier, cell death was broadly classified into only two distinct types: apoptosis and necrosis. However, in recent years, it has become increasingly evident that such a classification is an oversimplification. Although 12 different types of cell death have been described in the literature, they can be grouped into five major types: apoptosis, necrosis, autophagy, paraptosis, and autoschizis. Some forms of death are classified under one of these other headings. For example, anoikis and oncosis are forms of apoptosis (triggered by cell detachment) and necrosis, respectively. Because of overlap and shared signaling pathways among different death programs, it is difficult to devise exclusive definitions for each of these cell death programs.

Necrosis

Necrosis results from a variety of accidental and lethal actions by toxins or physical stimuli or in association with pathologic conditions, such as ischemia. Necrosis is characterized by cellular edema, lysis of nuclear chromatin, disruption of the plasma membrane, and loss of cellular contents into the extracellular space, resulting in inflammation (see Figure 4.1). In contrast, in the setting of apoptosis, membrane damage occurs late in the process, and dead cells are engulfed by neighboring cells or phagocytes, leading to little or no inflammation (see Figure 4.1). Although necrosis has mostly been regarded as an accidental form of cell death, more recent data have suggested that necrosis can also occur as a programmed form of cell death. There is growing evidence that necrotic and apoptotic forms of cell death may have similarities.[6]

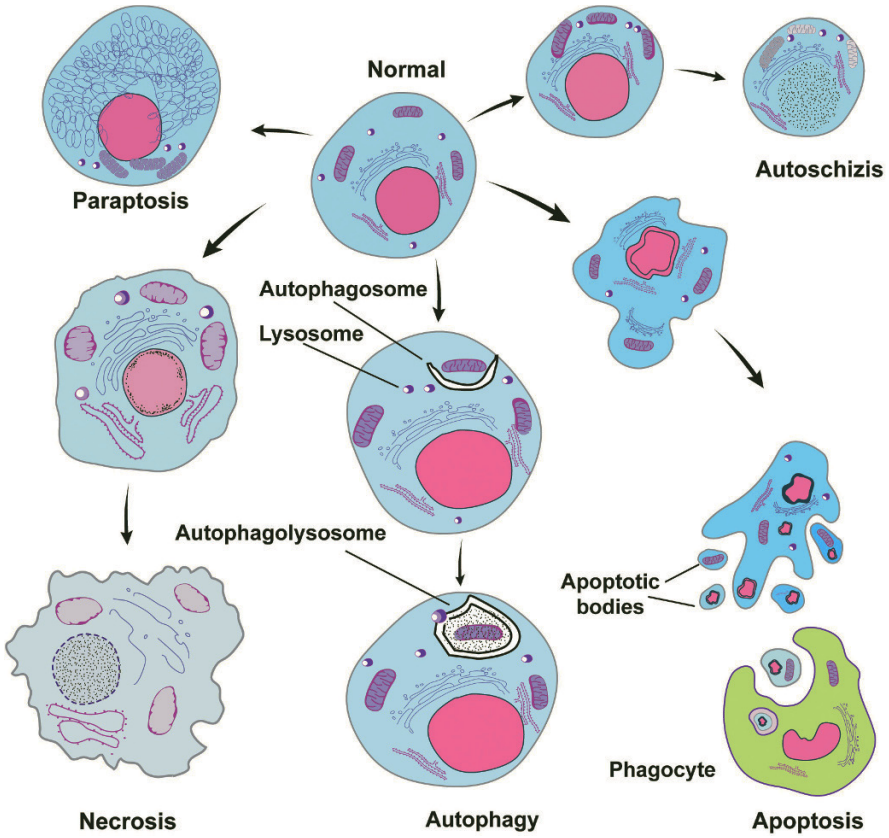

FIGURE 4.1. Morphologic features of cell death. *Necrosis*: Cells die by necrosis, and their organelles are characteristically swollen. There is early membrane damage with eventual loss of plasma membrane integrity and leakage of cytosol into extracellular space. Despite early clumping, the nuclear chromatin undergoes lysis (karyolysis). *Apoptosis*: Cells die by type I programmed cell death (also called apoptosis); they are shrunken and develop blebs containing dense cytoplasm. Membrane integrity is not lost until after cell death. Nuclear chromatin undergoes striking condensation and fragmentation. The cytoplasm becomes divided to form apoptotic bodies containing organelles and/or nuclear debris. Terminally, apoptotic cells and fragments are engulfed by phagocytes or surrounding cells. *Autophagy*: Cells die by type II programmed cell death, which is characterized by the accumulation of autophagic vesicles (autophagosomes and autophagolysosomes). One feature that distinguishes apoptosis from autophagic cell death is the source of the lysosomal enzymes used for most of the dying-cell degradation. Apoptotic cells use phagocytic cell lysosomes for this process, whereas cells with autophagic morphology use the endogenous lysosomal machinery of dying cells. *Paraptosis*: Cells die by type III programmed cell death, which is characterized by extensive cytoplasmic vacuolization and swelling and clumping of mitochondria, along with absence of nuclear fragmentation, membrane blebbing, or apoptotic body formation. *Autoschizis*: In this form of cell death, the cell membrane forms cuts or schisms that allow the cytoplasm to leak out. The cell shrinks to about one-third of its original size, and the nucleus and organelles remain surrounded by a tiny ribbon of cytoplasm. After further excisions of cytoplasm, the nuclei exhibit nucleolar segregation and chromatin decondensation followed by nuclear karyorrhexis and karyolysis.

Nevertheless, necrosis has been shown to occur in cells having defects in apoptotic machinery or upon inhibition of apoptosis,[7] and this form of cell death is emerging as an important therapeutic tool for cancer treatment.[8]

Autophagy

Autophagy, which is also referred to as *type II programmed cell death*, is characterized by sequestration of cytoplasm and organelles in double or multimembrane structures called *autophagic vesicles*, followed by degradation of the contents of these vesicles by the cell's own lysosomal system (see Figure 4.1). The precise role of autophagy in cell death or survival is not clearly understood. Autophagy has long been regarded as a cell survival mechanism whereby cells eliminate long-lived proteins and organelles. In this regard, it is argued that autophagy may help cancer cells survive under nutrient-limiting and low-oxygen conditions and against ionizing radiation.[9,10] However, recent observations that there is

decreased autophagy during experimental carcinogenesis and heterologous disruption of an autophagy gene, *Beclin 1* (Bcn1), in cancer cells[11,12] suggest that breakdown of autophagic machinery may contribute to development of cancer. Other interesting studies have shed some light on the relationship between autophagy and apoptosis. These investigations have shown prevention of caspase inhibitor z-VAD–induced cell death in mouse L929 cells by RNA interference directed against autophagy genes *atg7* and Bcn1[13] and protection of $Bax^{-/-}$, $Bak^{-/-}$ murine embryonic fibroblasts against staurosporine- or etoposide-induced cell death by RNA interference against autophagy genes *atg5* and Bcn1.[14] However, both of these studies were done in cells whose apoptotic pathways had been compromised. Thus, it remains to be seen whether cells with intact apoptotic machinery can also die by autophagy and whether apoptotic-competent cells lacking autophagy genes will be resistant to different death stimuli.

Paraptosis

Paraptosis has recently been described as a form of cell death characterized by extensive cytoplasmic vacuolation (see Figure 4.1) caused by swelling of mitochondria and endoplasmic reticulum. This form of cell death does not involve caspase activation, is not inhibited by caspase inhibitors, but is inhibited by the inhibitors of transcription and translation, actinomycin D, and cycloheximide, respectively,[15] suggesting a requirement for new protein synthesis. The tumor necrosis factor receptor family TAJ/TROY and the insulin-like growth factor I receptor have been shown to trigger paraptosis.[16] Paraptosis appears to be mediated by mitogen-activated protein kinases and inhibited by AIP1/Alix, a protein interacting with the calcium-binding death-related protein ALG-2.[16]

Autoschizis

Autoschizis is a recently described type of cell death that differs from apoptosis and necrosis and is induced by oxidative stress.[17] In this type of death, cells lose cytoplasm by self-morsellation or self-excision (see Figure 4.1). Autoschizis usually affects contiguous groups of cells both in vitro and in vivo but can also occasionally affect scattered individual cells trapped in subcapsular sinuses of lymph nodes.[18] The nuclear envelope and pores remain intact while the cytoplasm is reduced to a narrow rim surrounding the nucleus. The chromatin marginates along the nuclear membrane, and mitochondria and other organelles around the nucleus aggregate as a result of cytoskeletal damage and condensation of the cytosol. Interestingly, the rough endoplasmic reticulum is preserved until the late stages of autoschizis, in which cells fragment and the nucleolus becomes condensed and breaks into smaller fragments.[19] Eventually, the nuclear envelope and the remaining organelles dissipate with cell demise.

Apoptosis

Genetic studies in the nematode worm *C. elegans* led to the characterization of apoptosis. Activation of specific death genes during the development of this worm results in death of exactly 131 cells, leaving 959 cells intact.[2] Further studies revealed that apoptosis can be divided into three successive stages: (1) commitment phase, in which death is initiated by specific extracellular or intracellular signals; (2) execution phase; and (3) clean-up phase, in which dead cells are removed by other cells with eventual degradation of the dead cells in the lysosomes of phagocytic cells.[20] The apoptotic machinery is conserved through evolution from worm to human.[21] In *C. elegans*, execution of apoptosis is mediated by CED-3 and CED-4 proteins. Commitment to a death signal results in the activation of CED-3 by CED-4 binding. The CED-9 protein prevents activation of CED-3 by binding to CED-4.[22,23]

Mechanisms of Apoptosis

Caspases

Studies over the past decade have indicated that two distinct apoptotic pathways are followed in mammalian systems: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. The executioners in both intrinsic and extrinsic pathways of cell death are the caspases,[24] which are cysteine proteases with specificity to cleave their substrates after aspartic acid residues. The central role of caspases in apoptosis is underscored by the observation that apoptosis and all classic changes associated with apoptosis can be blocked by inhibition of caspase activity. To date, 12 mammalian caspases (caspase-1 to -10, caspase-14, and mouse caspase-12) have been identified.[25] Caspase-13 was later found to represent a bovine homolog and caspase-11 appears to be a murine homolog of human caspases-4 and -5, respectively.

Caspases are normally produced as inactive zymogens containing an N-terminal prodomain followed by a large and a small subunit that constitute the catalytic core of the protease. They have been categorized into two distinct classes: initiator and effector caspases. The upstream initiator caspases contain long N-terminal prodomains and one of the two characteristic protein–protein interaction motifs: the death effector domain (DED; caspase-8 and -10) and the caspase activation and recruitment

domain (caspase-1, -2, -4, -5, -9, and -12). The downstream effector caspases (caspase-3, -6, and -7) are characterized by the presence of a short prodomain. Apart from the structural differences, a prominent difference between initiator and effector caspases is their basal state. Both the zymogen and the activated forms of effector caspases exist as constitutive homodimers, whereas initiator caspase-9 exists predominantly as a monomer both before and after proteolytic processing.[26] Initiator caspase-8 has been reported to exist in an equilibrium between monomers and homodimers.[27] Although the initiator caspases are capable of autocatalytic activation, the activation of effector caspases requires formation of oligomeric complexes with their adapter proteins and often intrachain cleavage within the initiator caspase.

Caspases have also been divided into three categories based on substrate specificity.[28] Group I members (caspase-1, -4, and -5) have a substrate specificity for the WEHD sequence with high promiscuity; group II members (caspase-2, -3, and -7 and CED-3) prefer the DEXD sequence and have an absolute requirement for aspartate (D) at P4; and members of group III (caspase-6, -8, and -9 and the "aspase" granzyme B) have a preference for (I/L/V)EXD sequences. Several reports have suggested a role for group I members in inflammation and that of group II and III members in apoptotic signaling events.

Extrinsic Death Pathway

The extrinsic pathway involves binding of death ligands such as tumor necrosis factor-α (TNF-α), CD95 ligand (Fas ligand), and TNF-related apoptosis-inducing ligand (TRAIL) to their cognate cell surface receptors TNFR1, CD95/Fas, TRAIL-R1, TRAIL-R2, and the DR series of receptors,[29] resulting in the activation of initiator caspase-8 (also known as FADD-homologous ICE/CED-3-like protease or FLICE) and subsequent activation of effector caspase-3 (Figure 4.2).[30] The cytoplasmic domains of death receptors contain the "death domain," which plays a crucial role in transmitting the signal from the cell's surface to intracellular signaling molecules. Binding of the ligands to their cognate receptors results in receptor trimerization and recruitment of adapter proteins to the cell membrane, which involves homophilic interactions between death domains of the receptors and the adapter proteins. The adapter protein for the receptors TNFR1 and DR3 is TNFR-associated death domain protein (TRADD)[31] and that for Fas, TRAIL-R1, TRAIL-R2, and DR4 is Fas-associated death domain protein (FADD).[32]

The receptor/ligand and FADD complex in turn recruits caspase-8 to the activated receptor, resulting in the formation of death-inducing signaling complex (DISC) and subsequent activation of caspase-8 through oligomerization and self-cleavage. Depending on the cell type and/or apoptotic stimulus, caspase-8 can also be activated by caspase-6.[33] Activated caspase-8 then activates effector caspase-3. In some cell types, cleavage of caspase-3 by caspase-8 also requires a mitochondrial amplification loop involving cleavage of proapoptotic protein Bid by caspase-8 and its translocation to the mitochondrial membrane, triggering the release of apoptogenic proteins from mitochondria into cytosol (see Figure 4.2). In these cell types, overexpression of *Bcl-2* and *Bcl-xL* can block CD95-induced apoptosis.[34]

Tumor necrosis factor-α is produced by T cells and activated macrophages in response to infection. Although TNF-α–mediated signaling can be propagated through either TNFR1 or TNFR2 receptors, the majority of biologic functions are initiated by TNFR1.[35] Binding of TNF-α to TNFR1 causes release of inhibitory protein silencer of death domain protein (SODD) from TNFR1, which enables recruitment of adapter protein TRADD. Signaling induced by activation of TNFR1 or DR3 diverges at the level of TRADD. In one pathway, nuclear translocation of the transcription factor nuclear factor-κB (NF-κB) and activation of c-Jun N-terminal kinase (JNK) are initiated, which results in the induction of a number of proinflammatory and immunomodulatory genes.[36] In another pathway, TNF-α signaling is coupled to Fas signaling events through interaction of TRADD with FADD.[37] The TNFR1–TRADD complex can alternatively engage TRAF2 protein, resulting in activation of transcription factor c-Jun, which is involved in survival signaling. Furthermore, binding of receptor interaction protein to TNFR1 through TRADD results in activation of transcription factor NF-κB, which suppresses apoptosis through transcriptional upregulation of antiapoptotic molecules such as TRAF1, TRAF2, cIAP1, cIAP2, and FLIP. The FLICE-associated huge protein was identified to be a CED-4 homolog interacting with the DED of caspase-8 and was shown to modulate Fas-mediated activation of caspase-8.[38] Another class of protein, FLIP (FLICE inhibitory protein), was shown to block Fas-induced and TNF-α–induced DISC formation and subsequent activation of caspase-8.[39]

Cytotoxic T cells play a major role in vertebrate defense against viral infection.[40] They induce cell death in infected cells to prevent viral multiplication and spread of infection.[41] Cytotoxic T cells can kill their targets either by activating the Fas ligand/Fas pathway or by injecting granzyme B, a serine protease, into target cells. Cytotoxic T cells carry Fas ligand on their surface but also carry granules containing the channel-forming protein perforin and granzyme B. Upon recognizing the infected cells, the lymphocytes bind and secrete granules onto the surface of infected cells. Perforin then assembles into transmembrane channels to allow the entry of granzyme B into the target cell. Upon entry, granzyme B, which cleaves after aspartate residues in proteins ("aspase"), activates one or

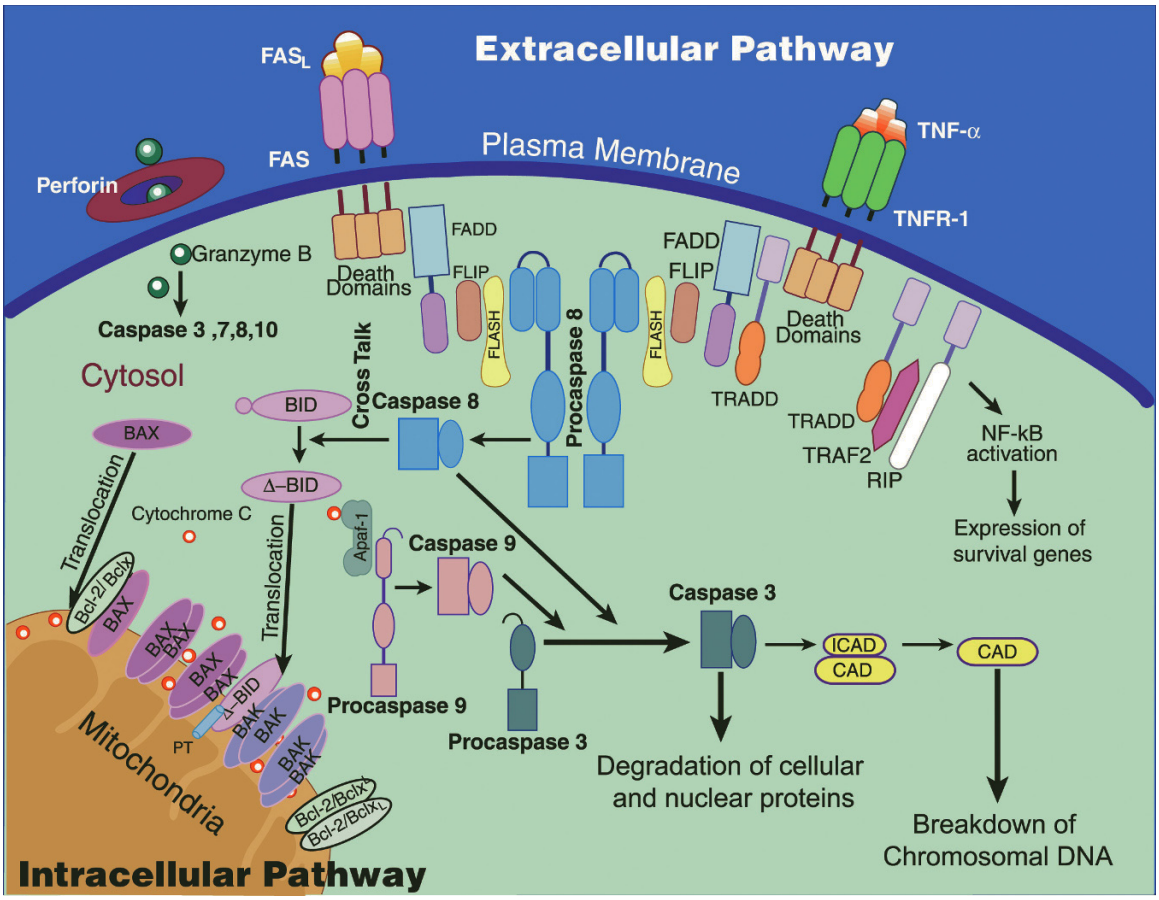

FIGURE 4.2. Schematic representation of apoptotic signaling pathways. *Extracellular Pathway:* Following the binding of peptides such as TNF-α or Fas ligand (FAS_L), the receptors oligomerize and recruit adapter proteins (Fas-associated death domain [FADD], tumor necrosis factor receptor [TNFR]–associated death domain [TRADD]) to form death-inducing signaling complexes, causing the activation of the initiator caspase-8, which sequentially activates effector caspases (e.g., caspase-3). Other adapter proteins (FLASH), inhibitory proteins (FLIP), or proteins involved in survival pathways as well as death mechanisms (receptor interaction protein [RIP]) may participate in complex mechanisms that determine life or death. The TNF-α TNFR1 complex can also elicit an antiapoptotic response by recruiting TRAF2, which results in NF-κB–mediated upregulation of antiapoptotic genes. In cytotoxic T lymphocyte–induced death, granzyme B, which enters the cell through membrane channels formed by the protein perforin, activates caspases by cleaving them directly or indirectly. *Intracellular Pathways:* Lack of survival stimuli (withdrawal of growth factor, hypoxia, genotoxic substances, etc.) is thought to generate apoptotic signals through ill-defined mechanisms, which lead to translocation of proapoptotic proteins such as Bax to the outer mitochondrial membrane. In some cases, transcription mediated by p53 may be required to induce proteins such as Bax. Translocated Bax undergoes conformational changes in the outer membrane to form oligomeric structures (pores) that leak cytochrome c from mitochondria into the cytosol. Formation of a ternary complex of cytochrome c, the adapter protein Apaf-1, and the initiator caspase-9 results in the activation of caspase-9 followed by sequential activation of effector caspase(s) such as caspase-3 and others. The action of caspases, endonucleases, and possibly other enzymes leads to cellular disintegration. For example, the endonuclease CAD (caspase activated DNAse) becomes activated when it is released from its inhibitor ICAD upon cleavage of ICAD by an effector caspase. Antiapoptotic proteins such as Bcl-2 and Bcl-xL inhibit the membrane-permeabilizing effects of Bax and other proapoptotic proteins. Cross-talk between extra- and intracellular pathways occurs through caspase-8–mediated Bid cleavage, which yields a 15 kDa protein that migrates to mitochondria and releases cytochrome c, thereby setting in motion events that lead to apoptosis via caspase-9.

more of the apoptotic proteases (caspase-2, -3, -7, -8, and -10) to trigger the proteolytic death cascade (see Figure 4.2). Fas ligand/Fas and perforin/granzyme B systems are the main apoptotic machinery that regulates homeostasis in immune cell populations.

Intrinsic Death Pathway

Cells can respond to various stressful stimuli and metabolic disturbances by triggering apoptosis. Drugs, toxins, heat, radiation, hypoxia, and viral infections are some of

the stimuli known to activate death pathways. Cell death, however, is not necessarily inevitable after exposure to these agents, and the mechanisms determining the outcome of the injury are a topic of active interest. The current consensus appears to be that it is the intensity and the duration of the stimulus that determine the outcome. The stimulus must go beyond a threshold to commit cells to apoptosis. Although the exact mechanism used by each stimulus may be unique and different, a few broad patterns can be identified. For example, agents that damage DNA, such as ionizing radiation and certain xenobiotics, lead to activation of p53-mediated mechanisms that commit cells to apoptosis, at least in part through transcriptional upregulation of proapoptotic proteins.[42] Other stresses induce increased activity of stress-activated protein kinases, which result ultimately in apoptotic commitment.[43] These different mechanisms converge in the activation of caspases.

A cascade of caspases plays the central executioner role by cleaving various mammalian cytosolic and nuclear proteins that play roles in cell division, maintenance of cytoskeletal structure, DNA replication and repair, RNA splicing, and other cellular processes. This proteolytic carnage produces the characteristic morphologic changes of apoptosis. Once the caspase cascade is initiated, the process of cell death has crossed the point of no return.

The roles of various caspases in apoptotic pathways and their relative importance for animal development have been examined in genetic studies involving knockout of different caspase genes. A caspase-1 (interleukin [IL]-1b converting enzyme [ICE]) knockout study suggested that ICE plays an important role in inflammation by activating cytokines such as IL-1b and IL-18. However, caspase-1 was not required to mediate apoptosis under normal circumstances and did not have a major role during development.[44] Surprisingly, ischemic brain injury was significantly reduced in caspase-1 knockout mice compared with wild-type mice,[45] suggesting that inflammation may contribute to ischemic injury. Caspase-3 deficiency leads to impaired brain development and premature death. Also, functional caspase-3 is required for some typical hallmarks of apoptosis such as formation of apoptotic bodies, chromatin condensation, and DNA fragmentation in many cell types.[46] Lack of caspase-8 results in the death of embryos at day 11 with abnormal formation of the heart,[47] suggesting that caspase-8 is required for cell death during mammalian development. In support of this finding, knockout of FADD, which is required for caspase-8 activation, resulted in fetal death with signs of abdominal hemorrhage and cardiac failure.[48] Moreover, caspase-8–deficient cells did not die in response to signals from members of the TNF receptor family.[47] However, cells lacking either FADD or caspase-8, which are resistant to TNF-α–mediated or CD95-mediated death, are susceptible to chemotherapeutic drugs, serum deprivation, ceramide, γ-irradiation, and dexamethasone-induced killing.[48] In contrast, caspase-9 has a key role in apoptosis induced by intracellular activators, particularly those that cause DNA damage. Deletion of caspase-9 resulted in perinatal lethality, apoptotic failure in developing neurons, enlarged brains, and craniofacial abnormalities.[49] In caspase-9–deficient cells, caspase-3 was not activated, suggesting that caspase-9 is upstream of caspase-3 in the apoptotic cascade. As a consequence, caspase-9–deficient cells are resistant to dexamethasone or irradiation, whereas they retain their sensitivity to TNF-α–induced or CD95-induced death[49] because of the presence of caspase-8, the initiator caspase involved in death receptor signaling that can also activate caspase-3. Overall, these observations support the idea that different death signaling pathways converge on downstream effector caspases (see Figure 4.2). Indeed, caspase-3 is regarded as one of the key executioner molecules activated by apoptotic stimuli originating either at receptors for exogenous molecules or within cells through the action of drugs, toxins, or radiation.

Regulators of Caspases

In *C. elegans*, biochemical and genetic studies have indicated a role for CED-4 upstream of CED-3.[50] Upon receiving death commitment signals, CED-4 binds to pro-CED-3 and releases active CED-3.[50] However, when overexpressed, CED-9 can inhibit the activation of pro-CED-3 by binding to CED-4 and sequestering it away from pro-CED-3. Therefore, CED-3 and CED-4 are involved in activation of apoptosis, and CED-9 inhibits apoptosis. After the discovery of caspases as CED-3 homologs, a search for activators and inhibitors analogous to CED-4 and CED-9 led to the discovery of diverse mammalian regulators of apoptosis. The plethora of these molecules and their functional diversity allowed them to be classified into four broad categories: (1) adapter proteins, (2) the Bcl-2 family of regulators, (3) inhibitors of apoptosis (IAPs), and (4) other regulators.

Adapter Proteins

As stated earlier, two major pathways of apoptosis, involving either the initiator caspase-8 or the initiator caspase-9 (see Figure 4.2), have been recognized. Signaling by death receptors (CD95, TNFRI) occurs through a well-defined process of recruitment of caspase-8 to the death receptor by adapter proteins such as FADD. Recruitment occurs through interactions between the death domains that are present on both receptor and adapter proteins. Receptor-bound FADD then recruits caspase-8 through interactions between DEDs common to both caspase-8 and FADD forming a DISC. In the DISC, caspase-8 activation occurs through oligomerization and autocatalysis.

Activated caspase-8 then activates downstream caspase-3, culminating in apoptosis. The inhibitory protein, FLIP was shown to block Fas-induced and TNF-α–induced DISC formation and subsequent activation of caspase-8.[39] Of particular interest is cellular FLIP, which stimulates caspase-8 activation at physiologically relevant levels and inhibited apoptosis upon high ectopic expression.[51] Cellular FLIP contains two DEDs that can compete with caspase-8 for recruitment to the DISC. This limits the degree of association of caspase-8 with FADD and thus limits activation of the caspase cascade. It also forms a heterodimer with caspase-8 and caspase-10 through interactions between both the DEDs and the caspase-like domains of the proteins, thus activating both caspase-8 and caspase-10.[52]

Apoptotic protease activating factor-1 (Apaf-1), a CED-4 homolog in mammalian cells, affects the activation of initiator caspase-9.[53] This factor binds to procaspase-9 in the presence of cytochrome c and 2′-deoxyadenosine 5′-triphosphate (dATP) or adenosine triphosphate (ATP) and activates this protease, which in turn activates a downstream cascade of proteases (see Figure 4.2).[54] By and large, Apaf-1 deficiency is embryonically lethal and the embryos exhibit brain abnormalities similar to those seen in caspase-9 knockout mice.[55] These genetic findings support the idea that Apaf-1 is coupled to caspase-9 in the death pathway. Unlike CED-4 in nematodes, Apaf-1 requires the binding of ATP and cytochrome c to activate procaspase-9. The multiple WD40 repeats in the C-terminal end of Apaf-1 have a regulatory role in the activation of caspase-9.[56]

The Bcl-2 Family of Proteins

The CED-9 homolog in mammals is the Bcl-2 protein. Bcl-2 was first discovered in B-cell lymphoma as a proto-oncogene. Overexpression of Bcl-2 was shown to offer protection against a variety of death stimuli.[57] The Bcl-2 protein family includes both proapoptotic (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Nr13, and A1/Bfl-1) and antiapoptotic proteins (Bax, Bak, Bok, Diva, Bcl-Xs, Bik, Bim, Hrk, Nip3, Nix, Bad, and Bid).[58] These proteins are characterized by the presence of Bcl-2 homology (BH) domains: BH1, BH2, BH3, and BH4 (Figure 4.3). The proapoptotic members have two subfamilies: a multidomain and a BH3-only group (see Figure 4.3). The relative ratio of pro- and antiapoptotic proteins determines the sensitivity of cells to various apoptotic stimuli.

The best-studied proapoptotic members are Bax and Bid. Exposure to various apoptotic stimuli leads to translocation of cytosolic Bax from the cytosol to the mitochondrial membrane.[59] Bax oligomerizes on the mitochondrial membrane along with another proapoptotic protein, Bak, leading to the release of cytochrome c from the mitochondrial membrane into the cytosol.[60]

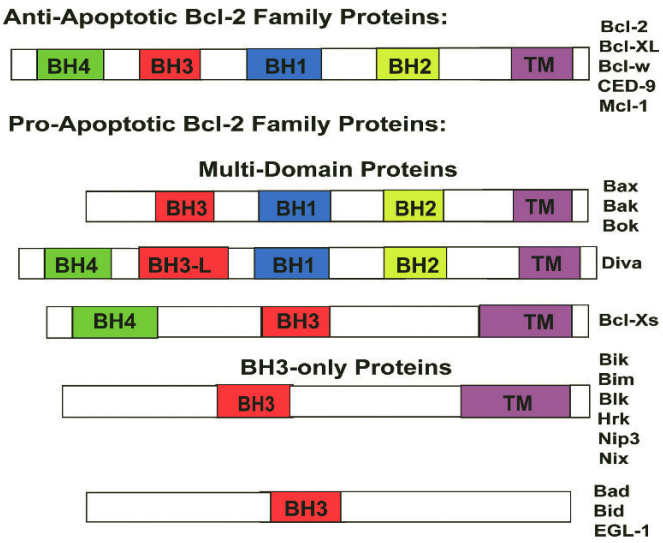

FIGURE 4.3. Structural homologies in anti- and proapoptotic proteins of the Bcl-2 family. Anti- and proapoptotic proteins of the Bcl-2 family proteins are depicted, indicating Bcl-2 homologous (BH) regions BH1, BH2, BH3, and BH4 and transmembrane (TM) domains.

Other proapoptotic proteins, mainly the BH3-only proteins, are thought to aid in Bax–Bak oligomerization on the mitochondrial membrane. The antiapoptotic Bcl-2 family members are known to block Bax–Bak oligomerization on the mitochondrial membrane and subsequent release of cytochrome c into the cytosol.[60,61] After release from the mitochondria, cytochrome c is known to interact with the WD40 repeats of the adaptor protein Apaf-1, resulting in the formation of the apoptosome complex.

Seven molecules of Apaf-1, interacting through their N-terminal caspase activation and recruitment domain, form the central hub region of the symmetric wheel-like structure, the apoptosome. Binding of ATP/dATP to Apaf-1 triggers the formation of the apoptosome, which subsequently recruits procaspase-9 into the apoptosome complex, resulting in its activation[62]. Activated caspase-9 then activates executioner caspases, such as caspase-3 and caspase-7, eventually leading to programmed cell death.

Inhibitors of Apoptosis Proteins

The IAPs, first discovered in baculoviruses and then in insects and *Drosophila*, inhibit activated caspases by directly binding to the active enzymes.[63] These proteins contain one or more baculovirus inhibitor of apoptosis repeat domains, which are responsible for the caspase inhibitory activity.[64] To date, eight mammalian IAPs have been identified. They include X-linked IAP (XIAP),

c-IAP1, c-IAP2, Melanoma IAP (ML-IAP)/Livin, IAP-like protein-2 (ILP-2), neuronal apoptosis-inhibitory protein (NAIP), Bruce/Apollon, and Survivin. In mammals, caspase-3, -7, and -9 are inhibited by IAPs.[62] There are reports suggesting aberrant expression of IAPs in many cancer tissues. For example, cIAP1 is overexpressed in esophageal squamous cell sarcoma[65]; cIAP2 locus is translocated in mucosa-associate lymphoid lymphoma[66] and Survivin has been shown to be upregulated in many cancer cells.[67]

Other Regulators

The caspase inhibitory activity of IAPs is inhibited by proteins containing an IAP-binding tetrapeptide motif.[62] The founding member of this family is SMAC/DIABLO, which is released from the mitochondrial intermembrane space into the cytosol during apoptosis. In the cytosol, it interacts with several IAPs and inhibits their function. The other mitochondrial protein, Omi/HtrA2, is also known to antagonize XIAP-mediated inhibition of caspase-9 at high concentrations.[68] A serine protease, Omi/HtrA2 can proteolytically cleave and inactivate IAP proteins and thus is considered to be a more potent suppressor of IAPs than SMAC.[69]

It has been reported that the heat shock proteins Hsp90, Hsp70, and Hsp27 can inhibit caspase activation by cytochrome c either by interacting with Apaf-1 or other players in the pathway.[70–72] A high-throughput screen identified a compound called PETCM (α-[trichloromethyl]-4-pyridineethanol) as a caspase-3 activator. Further work with PETCM revealed its involvement in apoptosome regulation.[73] This pathway also includes oncoprotein prothymosin-α and tumor suppressor putative HLA-DR–associated proteins. These proteins were shown to promote caspase-9 activation after apoptosome formation, whereas prothymosin-α inhibited caspase-9 activation by inhibiting apoptosome formation.

Protein Targets of Caspases

In an apoptotic cell, the regulatory, structural, and housekeeping proteins are the main targets of the caspases. The regulatory proteins mitogen-activated protein/extracellular signal-regulated kinase kinase-1, p21-activated kinase-2, and Mst-1 are activated upon cleavage by caspases.[74] Caspase-mediated protein hydrolysis inactivates other proteins, including focal adhesion kinase, phosphatidylinositol-3 kinase, Akt, Raf-1, IAPs, and inhibitors of caspase-activated DNAse (ICAD). Caspases also convert the antiapoptotic protein Bcl-2 into a proapoptotic protein such as Bax upon cleavage. There are many structural protein targets of caspases, which include nuclear lamins, actin, and regulatory proteins such as spectrin, gelsolin, and fodrins.[75]

Degradation of nuclear DNA into internucleosomal chromatin fragments is one of the hallmarks of apoptotic cell death that occurs in response to various apoptotic stimuli in a wide variety of cells. A specific DNase, CAD (caspase-activated DNase), that cleaves chromosomal DNA in a caspase-dependent manner, is synthesized with the help of ICAD. In proliferating cells, CAD is always found to be associated with ICAD in the cytosol. When cells are undergoing apoptosis, caspases (particularly caspase-3) cleave ICAD to release CAD and allow its translocation to the nucleus to cleave chromosomal DNA. Thus, cells that are ICAD deficient or that express caspase-resistant ICAD mutant do not exhibit DNA fragmentation during apoptosis.

Apoptosis and the Pathogenesis of Lung Diseases

Apoptosis plays a critical role in the postnatal lung.[76] Regulated removal of inflammatory cells by apoptosis helps in the resolution of inflammation in the lung.[77] Recent evidence also supports a role for apoptosis in the remodeling of lung tissue after acute lung injury[78] and in the pathogenesis of chronic pulmonary hypertension,[79] idiopathic pulmonary fibrosis, and chronic obstructive pulmonary disease.[80,81]

Acute Lung Injury/Acute Respiratory Distress Syndrome

Acute lung injury, which clinically manifests itself as the acute respiratory distress syndrome (ARDS), involves disruption of the alveolar epithelium and endothelium, increased vascular permeability, and edema. Two main hypotheses link the pathogenesis of ARDS to apoptosis, namely, the "neutrophilic hypothesis" and the "epithelial hypothesis." These two hypotheses are not mutually exclusive, and both could play important roles in the pathogenesis of ARDS.

The neutrophilic hypothesis suggests that neutrophil apoptosis plays an important role in the resolution of inflammation and that the inhibition of neutrophil apoptosis or the inhibition of clearance of apoptotic neutrophils is deleterious in ARDS.[82,83] Studies in humans showed that bronchoalveolar lavage fluids from patients with early ARDS inhibit the rate at which neutrophils develop apoptosis in vitro.[84] The inhibitory effect of bronchoalveolar lavage fluids on neutrophil apoptosis is mediated by granulocyte/macrophage colony-stimulating factor, and possibly by IL-8 and IL-2.[85,86] A membrane surface molecule, CD44, has been shown to play an important role in the clearance of apoptotic cells in vivo and in vitro.[87] In a model of bleomycin-induced lung

injury, CD44-deficient mice failed to clear apoptotic neutrophils, which was associated with worsened inflammation and increased mortality.[87] Activation of phagocytic cells inhibits production of proinflammatory cytokines, including IL-1β, IL-8, IL-10, granulocyte/macrophage colony-stimulating factor, and TNF-α and increases release of anti-inflammatory mediators such as transforming growth factor-β, prostaglandin E_2, and platelet-activating factor.[88,89] The net effects of these changes could favor resolution of inflammation.

The epithelial hypothesis suggests that the apoptotic death of alveolar epithelial cells, in response to soluble mediators such as Fas ligand, contributes to the prominent alveolar epithelial injury characteristic of ARDS. Several lines of evidence suggest a role for the Fas/Fas ligand system in epithelial cell apoptosis.[90] Fas is expressed on alveolar and airway epithelial cells,[91,92] and its expression increases in response to inflammatory mediators such as lipopolysaccharide. Fas-mediated lung cell apoptosis is modulated by surfactant protein A, which inhibits apoptosis in vivo.[93]

Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease, caused primarily by smoking, generally refers to chronic bronchitis and emphysema. Several factors, including protease/antiprotease imbalance, oxidative stress, cigarette smoke–derived toxins, and inflammation mediated by neutrophils, macrophages, and $CD8^+$ T cells, have been shown to contribute to the disease process. Furthermore, matrix metalloproteinase[94] and vascular endothelial growth factor receptor inhibition,[95,96] but not Fas/Fas ligand, have been shown to play role in the development of emphysema.

Asthma

Allergic asthma is characterized by intermittent or persistent bronchoconstriction and has been linked to airway remodeling and chronic inflammation, with increased numbers of eosinophils, $CD4^+$ T cells, and mast cells. Although at present a role for apoptosis in asthma is not confirmed, studies ex vivo have shown reduced apoptosis of circulating peripheral $CD4^+$ T cells and eosinophils in asthma, which might contribute to inflammation. Corticosteroids used to reduce inflammation in asthma have been shown to induce eosinophil apoptosis.[97]

Pulmonary Fibrosis

Pulmonary fibrosis is characterized by epithelial damage, fibroblast proliferation, and deposition of collagen. Although the mechanism of alveolar epithelial cell apoptosis in pulmonary fibrosis is not known, several reports have suggested Fas pathway,[98] angiotensin pathway,[99] activated T cell-derived perforin,[100] IL-13 stimulation,[101] and transforming growth factor-β1 activation[102] to play critical roles.

Lung Cancer

Because insufficient apoptosis is often associated with tumorigenesis, modulation of apoptotic and antiapoptotic targets seems to be an attractive approach to cancer therapy. Lung cancers can be divided into small cell lung cancers (SCLCs) and non–small cell lung cancers (NSCLCs).[103] The SCLCs are relatively more sensitive to anticancer drugs and irradiation than are the NSCLCs,[104] but the molecular basis for this difference is not clearly known. Evaluation of apoptosis-associated substances has shown that caspase-8, Fas, and Fas ligand are often downregulated in SCLCs but not in NSCLCs.[105] An investigation of the basis for these differences revealed that there were no differences in the levels of Bax and Bcl-xL, but the expression of Bcl-2 was found to be significantly higher in SCLC than in NSCLC cell lines. The observation that in some cases Bcl-2 can be converted into a proapoptotic Bax-like death molecule may offer an explanation for the paradoxic expression of Bcl-2 in SCLC.[106] The lack of expression of procaspase-1, -4, -8, and -10[107] reported in SCLC suggests that these caspases probably do not contribute to spontaneous apoptosis in these cells. Apoptosis regulators Apaf-1 and procaspase-3 are overexpressed and are functional in NSCLC cell lines. In both types of lung cancer, apoptotic stimuli result in cytochrome c release and activation of caspase-9 and caspase-3, but only SCLC cell lines showed a relocalization of caspase-3 into the nucleus[108]; this suggests that the resistance of NSCLC cell lines is probably due to defective relocalization of caspase-3. The expression of caspase-9 and caspase-7 in NSCLCs was found to be similar to normal lung tissue.[109] However, these cell lines express the apoptosis inhibitor and splice variant of caspase-9 CASP9b. In vitro, chemotherapy-resistant NSCLC cell lines exhibit decreased caspase-9 and caspase-3 expression,[110] which suggests an inhibition of apoptosis induction via apoptosome formation in NSCLC.

Additionally, both NSCLC and SCLC cells express high and almost equal levels of Survivin.[107] The resistant NSCLC cells showed higher expression of c-IAP2, and the radiosensitive SCLC cells exhibited increased expression of XIAP.[111] These results suggest no correlation between the level of expression of the IAPs and the difference in the radiosensitivity between NSCLC and SCLC cells.

Conclusion

Cell death has become an area of intense interest and investigation in science and medicine because of the recognition that cell death, in general, and apoptosis, in par-

ticular, are important features of many biologic processes. Involvement of many genes in the death process suggests that cell death is a complex phenomenon with many redundant mechanisms to ensure definitiveness. The realization that defective cell death plays a central role in the pathogenesis of diseases has stimulated work on therapies targeted to these processes, and this work will undoubtedly continue in the future.

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