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Apoptosis: A Review of Programmed Cell Death

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Abstract

The process of programmed cell death, or apoptosis, is generally characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms. Apoptosis is considered a vital component of various processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death. Inappropriate apoptosis (either too little or too much) is a factor in many human conditions including neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer. The ability to modulate the life or death of a cell is recognized for its immense therapeutic potential. Therefore, research continues to focus on the elucidation and analysis of the cell cycle machinery and signaling pathways that control cell cycle arrest and apoptosis. To that end, the field of apoptosis research has been moving forward at an alarmingly rapid rate. Although many of the key apoptotic proteins have been identified, the molecular mechanisms of action or inaction of these proteins remain to be elucidated. The goal of this review is to provide a general overview of current knowledge on the process of apoptosis including morphology, biochemistry, the role of apoptosis in health and disease, detection methods, as well as a discussion of potential alternative forms of apoptosis.

Keywords

Apoptosis; programmed cell death; intrinsic/extrinsic pathway; granzyme A/B; perforin; autophagy

Introduction

The term apoptosis (a-po-toe-sis) was first used in a now-classic paper by Kerr, Wyllie, and Currie in 1972 to describe a morphologically distinct form of cell death, although certain components of the apoptosis concept had been explicitly described many years previously (Kerr et al., 1972; Paweletz, 2001; Kerr, 2002). Our understanding of the mechanisms involved in the process of apoptosis in mammalian cells transpired from the investigation of programmed cell death that occurs during the development of the nematode *Caenorhabditis elegans* (Horvitz, 1999). In this organism 1090 somatic cells are generated in the formation of the adult worm, of which 131 of these cells undergo apoptosis or "programmed cell death." These 131 cells die at particular points during the development process, which is essentially invariant between worms, demonstrating the remarkable accuracy and control in this system. Apoptosis has since been recognized and accepted as a distinctive and important mode of "programmed" cell death, which involves the genetically determined elimination of cells. However, it is important to note that other forms of programmed cell death have been described and other forms of programmed cell death may yet be discovered (Formigli et al., 2000; Sperandio et al., 2000; Debnath et al., 2005).

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Apoptosis occurs normally during development and aging and as a homeostatic mechanism to maintain cell populations in tissues. Apoptosis also occurs as a defense mechanism such as in immune reactions or when cells are damaged by disease or noxious agents (Norbury and Hickson, 2001). Although there are a wide variety of stimuli and conditions, both physiological and pathological, that can trigger apoptosis, not all cells will necessarily die in response to the same stimulus. Irradiation or drugs used for cancer chemotherapy results in DNA damage in some cells, which can lead to apoptotic death through a *p53*-dependent pathway. Some hormones, such as corticosteroids, may lead to apoptotic death in some cells (e.g., thymocytes) although other cells are unaffected or even stimulated.

Some cells express Fas or TNF receptors that can lead to apoptosis via ligand binding and protein cross-linking. Other cells have a default death pathway that must be blocked by a survival factor such as a hormone or growth factor. There is also the issue of distinguishing apoptosis from necrosis, two processes that can occur independently, sequentially, as well as simultaneously (Hirsch, 1997; Zeiss, 2003). In some cases it's the type of stimuli and/or the degree of stimuli that determines if cells die by apoptosis or necrosis. At low doses, a variety of injurious stimuli such as heat, radiation, hypoxia and cytotoxic anticancer drugs can induce apoptosis but these same stimuli can result in necrosis at higher doses. Finally, apoptosis is a coordinated and often energy-dependent process that involves the activation of a group of cysteine proteases called "caspases" and a complex cascade of events that link the initiating stimuli to the final demise of the cell.

Morphology of Apoptosis

Light and electron microscopy have identified the various morphological changes that occur during apoptosis (Hacker, 2000). During the early process of apoptosis, cell shrinkage and pyknosis are visible by light microscopy (Kerr et al., 1972). With cell shrinkage, the cells are smaller in size, the cytoplasm is dense and the organelles are more tightly packed. Pyknosis is the result of chromatin condensation and this is the most characteristic feature of apoptosis. On histologic examination with hematoxylin and eosin stain, apoptosis involves single cells or small clusters of cells. The apoptotic cell appears as a round or oval mass with dark eosinophilic cytoplasm and dense purple nuclear chromatin fragments (Figure 1). Electron microscopy can better define the subcellular changes. Early during the chromatin condensation phase, the electron-dense nuclear material characteristically aggregates peripherally under the nuclear membrane although there can also be uniformly dense nuclei (Figures 2A, 2B).

Extensive plasma membrane blebbing occurs followed by karyorrhexis and separation of cell fragments into apoptotic bodies during a process called "budding." Apoptotic bodies consist of cytoplasm with tightly packed organelles with or without a nuclear fragment (Figure 2C). The organelle integrity is still maintained and all of this is enclosed within an intact plasma membrane. These bodies are subsequently phagocytosed by macrophages, parenchymal cells, or neoplastic cells and degraded within phagolysosomes (Figure 2D). Macrophages that engulf and digest apoptotic cells are called "tingible body macrophages" and are frequently found within the reactive germinal centers of lymphoid follicles or occasionally within the thymic cortex. The tingible bodies are the bits of nuclear debris from the apoptotic cells. There is essentially no inflammatory reaction associated with the process of apoptosis nor with the removal of apoptotic cells because: (1) apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue; (2) they are quickly phagocytosed by surrounding cells thus likely preventing secondary necrosis; and, (3) the engulfing cells do not produce anti-inflammatory cytokines (Savill and Fadok, 2000; Kurosaka et al., 2003).

Distinguishing Apoptosis from Necrosis

The alternative to apoptotic cell death is necrosis, which is considered to be a toxic process where the cell is a passive victim and follows an energy-independent mode of death. But since necrosis refers to the degradative processes that occur after cell death, it is considered by some to be an inappropriate term to describe a mechanism of cell death. Oncosis is therefore used to describe a process that leads to necrosis with karyolysis and cell swelling whereas apoptosis leads to cell death with cell shrinkage, pyknosis, and karyorrhexis. Therefore the terms "oncotic cell death" and "oncotic necrosis" have been proposed as alternatives to describe cell death that is accompanied by cell swelling, but these terms are not widely used at this time (Majno and Joris, 1995; Levin et al., 1999).

Although the mechanisms and morphologies of apoptosis and necrosis differ, there is overlap between these two processes. Evidence indicates that necrosis and apoptosis represent morphologic expressions of a shared biochemical network described as the "apoptosis-necrosis continuum" (Zeiss, 2003). For example, two factors that will convert an ongoing apoptotic process into a necrotic process include a decrease in the availability of caspases and intracellular ATP (Leist et al., 1997; Denecker et al., 2001). Whether a cell dies by necrosis or apoptosis depends in part on the nature of the cell death signal, the tissue type, the developmental stage of the tissue and the physiologic milieu (Fiers et al., 1999; Zeiss, 2003).

Using conventional histology, it is not always easy to distinguish apoptosis from necrosis, and they can occur simultaneously depending on factors such as the intensity and duration of the stimulus, the extent of ATP depletion and the availability of caspases (Zeiss, 2003). Necrosis is an uncontrolled and passive process that usually affects large fields of cells whereas apoptosis is controlled and energy-dependent and can affect individual or clusters of cells. Necrotic cell injury is mediated by two main mechanisms; interference with the energy supply of the cell and direct damage to cell membranes.

Some of the major morphological changes that occur with necrosis include cell swelling; formation of cytoplasmic vacuoles; distended endoplasmic reticulum; formation of cytoplasmic blebs; condensed, swollen or ruptured mitochondria; disaggregation and detachment of ribosomes; disrupted organelle membranes; swollen and ruptured lysosomes; and eventually disruption of the cell membrane (Kerr et al., 1972; Majno and Joris, 1995; Trump et al., 1997). This loss of cell membrane integrity results in the release of the cytoplasmic contents into the surrounding tissue, sending chemotatic signals with eventual recruitment of inflammatory cells. Because apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue and are quickly phagocytosed by macrophages or adjacent normal cells, there is essentially no inflammatory reaction (Savill and Fadok, 2000; Kurosaka et al., 2003). It is also important to note that pyknosis and karyorrhexis are not exclusive to apoptosis and can be a part of the spectrum of cytomorphological changes that occurs with necrosis (Cotran et al., 1999). Table 1 compares some of the major morphological features of apoptosis and necrosis.

Is Apoptosis an Irreversible Process?

Many of the genes that control the killing and engulfment processes of programmed cell death have been identified, and the molecular mechanisms underlying these processes have proven to be evolutionarily conserved (Metzstein et al., 1998). Until recently, apoptosis has traditionally been considered an irreversible process with caspase activation committing a cell to death and the engulfment genes serving the purpose of dead cell removal. However, the uptake and clearance of apoptotic cells by macrophages may involve more than just the removal of cell debris. Hoeppner et al. have shown that blocking engulfment genes in *C. elegans*

embryos enhances cell survival when cells are subjected to weak pro-apoptotic signals (Hoeppner et al., 2001).

Reddien et al. demonstrated that, in *C. elegans*, mutations that cause partial loss of function of killer genes allow the survival of some cells that are programmed to die via apoptosis, and mutations in engulfment genes enhance the frequency of this cell survival. Moreover, mutations in engulfment genes alone allowed the survival and differentiation of some cells that were otherwise destined to die via apoptosis (Reddien et al., 2001). These findings suggest that genes that mediate corpse removal can also function to actively kill cells. In other words, the engulfing cells may act to ensure that cells triggered to undergo apoptosis will die rather than recover after the initial stages of death.

In vertebrates, there is some evidence of a potential role for macrophages in promoting the death of cells in some tissues. Elimination of macrophages in the anterior chamber of the rat eye resulted in the survival of vascular endothelial cells that normally undergo apoptosis (Diez-Roux and Lang, 1997). Other studies have demonstrated that inhibition of macrophages can disrupt the remodeling of tissues in the mouse eye or in the tadpole tail during regression; two processes that involve apoptosis (Lang and Bishop, 1993; Little and Flores, 1993). Geske and coworkers (2001) demonstrated that early p53-induced apoptotic cells can be rescued from the apoptotic program if the apoptotic stimulus is removed. Their research suggests that DNA repair is activated early in the *p*53-induced apoptotic process and that this DNA repair may be involved in reversing the cell death pathway in some circumstances.

Mechanisms of Apoptosis

The mechanisms of apoptosis are highly complex and sophisticated, involving an energy-dependent cascade of molecular events (Figure 3). To date, research indicates that there are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. However, there is now evidence that the two pathways are linked and that molecules in one pathway can influence the other (Igney and Krammer, 2002). There is an additional pathway that involves T-cell mediated cytotoxicity and perforin-granzyme-dependent killing of the cell. The perforin/granzyme pathway can induce apoptosis via either granzyme B or granzyme A. The extrinsic, intrinsic, and granzyme B pathways converge on the same terminal, or execution pathway. This pathway is initiated by the cleavage of caspase-3 and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells. The granzyme A pathway activates a parallel, caspase-independent cell death pathway via single stranded DNA damage (Martinvalet et al., 2005).

Biochemical Features—Apoptotic cells exhibit several biochemical modifications such as protein cleavage, protein cross-linking, DNA breakdown, and phagocytic recognition that together result in the distinctive structural pathology described previously (Hengartner, 2000). Caspases are widely expressed in an inactive proenzyme form in most cells and once activated can often activate other procaspases, allowing initiation of a protease cascade. Some procaspases can also aggregate and autoactivate. This proteolytic cascade, in which one caspase can activate other caspases, amplifies the apoptotic signaling pathway and thus leads to rapid cell death.

Caspases have proteolytic activity and are able to cleave proteins at aspartic acid residues, although different caspases have different specificities involving recognition of neighboring amino acids. Once caspases are initially activated, there seems to be an irreversible commitment towards cell death. To date, ten major caspases have been identified and broadly categorized into initiators (caspase-2,-8,-9,-10), effectors or executioners (caspase-3,-6,-7) and

inflammatory caspases (caspase-1,-4,-5) (Cohen, 1997; Rai et al., 2005). The other caspases that have been identified include caspase-11, which is reported to regulate apoptosis and cytokine maturation during septic shock, caspase-12, which mediates endoplasmic-specific apoptosis and cytotoxicity by amyloid-β, caspase-13, which is suggested to be a bovine gene, and caspase-14, which is highly expressed in embryonic tissues but not in adult tissues (Hu et al., 1998; Nakagawa et al., 2000, Koenig et al., 2001; Kang et al., 2002).

Extensive protein cross-linking is another characteristic of apoptotic cells and is achieved through the expression and activation of tissue transglutaminase (Nemes et al., 1996). DNA breakdown by Ca^{2+}-and Mg^{2+}-dependent endonucleases also occurs, resulting in DNA fragments of 180 to 200 base pairs (Bortner et al., 1995). A characteristic "DNA ladder" can be visualized by agarose gel electrophoresis with an ethidium bromide stain and ultraviolet illumination.

Another biochemical feature is the expression of cell surface markers that result in the early phagocytic recognition of apoptotic cells by adjacent cells, permitting quick phagocytosis with minimal compromise to the surrounding tissue. This is achieved by the movement of the normal inward-facing phosphatidylserine of the cell's lipid bilayer to expression on the outer layers of the plasma membrane (Bratton et al., 1997). Although externalization of phosphatidylserine is a well-known recognition ligand for phagocytes on the surface of the apoptotic cell, recent studies have shown that other proteins are also be exposed on the cell surface during apoptotic cell clearance. These include Annexin I and calreticulin.

Annexin V is a recombinant phosphatidylserine-binding protein that interacts strongly and specifically with phosphatidylserine residues and can be used for the detection of apoptosis (Van Engeland et al., 1998; Arur et al., 2003). Calreticulin is a protein that binds to an LDL-receptor related protein on the engulfing cell and is suggested to cooperate with phosphatidylserine as a recognition signal (Gardai et al., 2005). The adhesive glycoprotein, thrombospondin-1, can be expressed on the outer surface of activated microvascular endothelial cells and, in conjunction with CD36, caspase-3-like proteases and other proteins, induce receptor-mediated apoptosis (Jimenez et al., 2000).

Extrinsic Pathway—The extrinsic signaling pathways that initiate apoptosis involve transmembrane receptor-mediated interactions. These involve death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily (Locksley et al., 2001). Members of the TNF receptor family share similar cyteine-rich extracellular domains and have a cytoplasmic domain of about 80 amino acids called the "death domain" (Ashkenazi and Dixit, 1998). This death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways. To date, the best-characterized ligands and corresponding death receptors include FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 (Chicheportiche et al., 1997; Ashkenazi et al., 1998; Peter and Kramer, 1998; Suliman et al., 2001; Rubio-Moscardo et al., 2005).

The sequence of events that define the extrinsic phase of apoptosis are best characterized with the FasL/FasR and TNF-α/TNFR1 models. In these models, there is clustering of receptors and binding with the homologous trimeric ligand. Upon ligand binding, cytplasmic adapter proteins are recruited which exhibit corresponding death domains that bind with the receptors. The binding of Fas ligand to Fas receptor results in the binding of the adapter protein FADD and the binding of TNF ligand to TNF receptor results in the binding of the adapter protein TRADD with recruitment of FADD and RIP (Hsu et al., 1995; Grimm et al., 1996; Wajant, 2002). FADD then associates with procaspase-8 via dimerization of the death effector domain. At this point, a death-inducing signaling complex (DISC) is formed, resulting in the auto-catalytic activation of procaspase-8 (Kischkel et al., 1995).

Once caspase-8 is activated, the execution phase of apoptosis is triggered. Death receptor-mediated apoptosis can be inhibited by a protein called c-FLIP which will bind to FADD and caspase-8, rendering them ineffective (Kataoka et al., 1998; Scaffidi, 1999). Another point of potential apoptosis regulation involves a protein called Toso, which has been shown to block Fas-induced apoptosis in T cells via inhibition of caspase-8 processing (Hitoshi et al., 1998). Table 2 lists the major extrinsic pathway proteins with common abbreviations and some of the alternate nomenclature used for each protein.

Perforin/granzyme Pathway—T-cell mediated cytotoxicity is a variant of type IV hypersensitivity where sensitized CD8+ cells kill antigen-bearing cells. These cytotoxic T lymphocytes (CTLs) are able to kill target cells via the extrinsic pathway and the FasL/FasR interaction is the predominant method of CTL-induced apoptosis (Brunner et al., 2003). However, they are also able to exert their cytotoxic effects on tumor cells and virus-infected cells via a novel pathway that involves secretion of the transmembrane pore-forming molecule perforin with a subsequent exophytic release of cytoplasmic granules through the pore and into the target cell (Trapani and Smyth, 2002). The serine proteases granzyme A and granzyme B are the most important component within the granules (Pardo et al., 2004).

Granzyme B will cleave proteins at aspartate residues and will therefore activate procaspase-10 and can cleave factors like ICAD (Inhibitor of Caspase Activated DNAse) (Sakahira et al., 1998). Reports have also shown that granzyme B can utilize the mitochondrial pathway for amplification of the death signal by specific cleavage of Bid and induction of cytochrome *c* release (Barry and Bleackley, 2002; Russell and Ley, 2002). However, granzyme B can also directly activate caspase-3. In this way, the upstream signaling pathways are bypassed and there is direct induction of the execution phase of apoptosis.

It is suggested that both the mitochondrial pathway and direct activation of caspase-3 are critical for granzyme B-induced killing (Goping et al., 2003). Recent findings indicate that this method of granzyme B cytotoxicity is critical as a control mechanism for T cell expansion of type 2 helper T (Th2) cells (Devadas et al., 2006). Moreover, findings indicate that neither death receptors nor caspases are involved with the T cell receptor-induced apoptosis of activated Th2 cells because blocking their ligands has no effect on apoptosis. On the other hand, Fas-Fas ligand interaction, adapter proteins with death domains and caspases are all involved in the apoptosis and regulation of cytotoxic Type 1 helper cells whereas granzyme B has no effect.

Granzyme A is also important in cytotoxic T cell induced apoptosis and activates caspase independent pathways. Once in the cell, granzyme A activates DNA nicking via DNAse NM23-H1, a tumor suppressor gene product (Fan et al., 2003). This DNAse has an important role in immune surveillance to prevent cancer through the induction of tumor cell apoptosis. The nucleosome assembly protein SET normally inhibits the NM23-H1 gene. Granzyme A protease cleaves the SET complex thus releasing inhibition of NM23-H1, resulting in apoptotic DNA degradation. In addition to inhibiting NM23-H1, the SET complex has important functions in chromatin structure and DNA repair. The proteins that make up this complex (SET, Ape1, pp32, and HMG2) seem to work together to protect chromatin and DNA structure (Lieberman and Fan, 2003). Therefore, inactivation of this complex by granzyme A most likely also contributes to apoptosis by blocking the maintenance of DNA and chromatin structure integrity.

Intrinsic Pathway—The intrinsic signaling pathways that initiate apoptosis involve a diverse array of non-receptor-mediated stimuli that produce intracellular signals that act directly on targets within the cell and are mitochondrial-initiated events. The stimuli that initiate the intrinsic pathway produce intracellular signals that may act in either a positive or negative fashion. Negative signals involve the absence of certain growth factors, hormones and

cytokines that can lead to failure of suppression of death programs, thereby triggering apoptosis. In other words, there is the withdrawal of factors, loss of apoptotic suppression, and subsequent activation of apoptosis. Other stimuli that act in a positive fashion include, but are not limited to, radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals.

All of these stimuli cause changes in the inner mitochondrial membrane that results in an opening of the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial transmembrane potential and release of two main groups of normally sequestered pro-apoptotic proteins from the intermembrane space into the cytosol (Saelens et al., 2004). The first group consists of cytochrome *c*, Smac/DIABLO, and the serine protease HtrA2/Omi (Cai et al., 1998; Du et al., 2000; Loo et al., 2002; Garrido et al., 2005). These proteins activate the caspase-dependent mitochondrial pathway. Cytochrome *c* binds and activates Apaf-1 as well as procaspase-9, forming an "apoptosome" (Chinnaiyan, 1999; Hill et al., 2004).

The clustering of procaspase-9 in this manner leads to caspase-9 activation. Smac/DIABLO and HtrA2/Omi are reported to promote apoptosis by inhibiting IAP (inhibitors of apoptosis proteins) activity (van Loo et al., 2002a; Schimmer, 2004). Additional mitochondrial proteins have also been identified that interact with and suppress the action of IAP however gene knockout experiments suggest that binding to IAP alone may not be enough evidence to label a mitochondrial protein as "pro-apoptotic" (Ekert and Vaux, 2005).

The second group of pro-apoptotic proteins, AIF, endonuclease G and CAD, are released from the mitochondria during apoptosis, but this is a late event that occurs after the cell has committed to die. AIF translocates to the nucleus and causes DNA fragmentation into ~50–300 kb pieces and condensation of peripheral nuclear chromatin (Joza et al., 2001). This early form of nuclear condensation is referred to as "stage I" condensation (Susin et al., 2000). Endonuclease G also translocates to the nucleus where it cleaves nuclear chromatin to produce oligonucleosomal DNA fragments (Li et al., 2001). AIF and endonuclease G both function in a caspase-independent manner. CAD is subsequently released from the mitochondria and translocates to the nucleus where, after cleavage by caspase-3, it leads to oligonucleosomal DNA fragmentation and a more pronounced and advanced chromatin condensation (Enari et al., 1998). This later and more pronounced chromatin condensation is referred to as "stage II" condensation (Susin et al., 2000).

The control and regulation of these apoptotic mitochondrial events occurs through members of the Bcl-2 family of proteins (Cory and Adams, 2002). The tumor suppressor protein *p53* has a critical role in regulation of the Bcl-2 family of proteins, however the exact mechanisms have not yet been completely elucidated (Schuler and Green, 2001). The Bcl-2 family of proteins governs mitochondrial membrane permeability and can be either pro-apoptotic or anti-apoptotic. To date, a total of 25 genes have been identified in the Bcl-2 family. Some of the anti-apoptotic proteins include Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG, and some of the pro-apoptotic proteins include Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk. These proteins have special significance since they can determine if the cell commits to apoptosis or aborts the process. It is thought that the main mechanism of action of the Bcl-2 family of proteins is the regulation of cytochrome *c* release from the mitochondria via alteration of mitochondrial membrane permeability.

A few possible mechanisms have been studied but none have been proven definitively. Mitochondrial damage in the Fas pathway of apoptosis is mediated by the caspase-8 cleavage of Bid (Li et al., 1998; Esposti, 2002). This is one example of the "cross-talk" between the death-receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway (Igney and Krammer, 2002). Serine phosphorylation of Bad is associated with 14-3-3, a member of a family of multifunctional phosphoserine binding molecules. When Bad is phosphorylated, it

is trapped by 14-3-3 and sequestered in the cytosol but once Bad is unphosphorylated, it will translocate to the mitochondria to release cytochrome C (Zha, et al., 1996).

Bad can also heterodimerize with Bcl-Xl or Bcl-2, neutralizing their protective effect and promoting cell death (Yang et al., 1995). When not sequestered by Bad, both Bcl-2 and Bcl-Xl inhibit the release of cytochrome C from the mitochondria although the mechanism is not well understood. Reports indicate that Bcl-2 and Bcl-XL inhibit apoptotic death primarily by controlling the activation of caspase proteases (Newmeyer et al., 2000). An additional protein designated "Aven" appears to bind both Bcl-Xl and Apaf-1, thereby preventing activation of procaspase-9 (Chau et al., 2000). There is evidence that overexpression of either Bcl-2 or Bcl-Xl will down-regulate the other, indicating a reciprocal regulation between these two proteins.

Puma and Noxa are two members of the Bcl2 family that are also involved in pro-apoptosis. Puma plays an important role in *p53*-mediated apoptosis. It was shown that, in vitro, overexpression of Puma is accompanied by increased BAX expression, BAX conformational change, translocation to the mitochondria, cytochrome *c* release and reduction in the mitochondrial membrane potential (Liu et al., 2003). Noxa is also a candidate mediator of *p53*-induced apoptosis. Studies show that this protein can localize to the mitochondria and interact with anti-apoptotic Bcl-2 family members, resulting in the activation of caspase-9 (Oda et al., 2000). Since both Puma and Noxa are induced by *p53*, they might mediate the apoptosis that is elicited by geno-toxic damage or oncogene activation. The Myc oncoprotein has also been reported to potentiate apoptosis through both *p53*-dependent and -independent mechanisms (Meyer et al., 2006).

Further elucidation of these pathways should have important implications for tumorigenesis and therapy. Table 3 lists the major intrinsic pathway proteins with common abbreviations and some of the alternate nomenclature used for each protein.

Execution Pathway

The extrinsic and intrinsic pathways both end at the point of the execution phase, considered the final pathway of apoptosis. It is the activation of the execution caspases that begins this phase of apoptosis. Execution caspases activate cytoplasmic endonuclease, which degrades nuclear material, and proteases that degrade the nuclear and cytoskeletal proteins. Caspase-3, caspase-6, and caspase-7 function as effector or "executioner" caspases, cleaving various substrates including cytokeratins, PARP, the plasma membrane cytoskeletal protein alpha fodrin, the nuclear protein NuMA and others, that ultimately cause the morphological and biochemical changes seen in apoptotic cells (Slee et al., 2001).

Caspase-3 is considered to be the most important of the executioner caspases and is activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10). Caspase-3 specifically activates the endonuclease CAD. In proliferating cells CAD is complexed with its inhibitor, ICAD. In apoptotic cells, activated caspase-3 cleaves ICAD to release CAD (Sakahira et al., 1998). CAD then degrades chromosomal DNA within the nuclei and causes chromatin condensation. Caspase-3 also induces cytoskeletal reorganization and disintegration of the cell into apoptotic bodies. Gelsolin, an actin binding protein, has been identified as one of the key substrates of activated caspase-3.

Gelsolin will typically act as a nucleus for actin polymerization and will also bind phosphatidylinositol biphosphate, linking actin organization and signal transduction. Caspase-3 will cleave gelsolin and the cleaved fragments of gelsolin, in turn, cleave actin filaments in a calcium independent manner. This results in disruption of the cytoskeleton, intracellular transport, cell division, and signal transduction (Kothakota et al., 1997).

Phagocytic uptake of apoptotic cells is the last component of apoptosis. Phospholipid asymmetry and externalization of phosphatidylserine on the surface of apoptotic cells and their fragments is the hallmark of this phase. Although the mechanism of phosphatidylserine translocation to the outer leaflet of the cell during apoptosis is not well understood, it has been associated with loss of aminophospholipid translocase activity and nonspecific flip-flop of phospholipids of various classes (Bratton et al., 1997). Research indicates that Fas, caspase-8, and caspase-3 are involved in the regulation of phosphatidylserine externalization on oxidatively stressed erythrocytes however caspase-independent phosphatidylserine exposure occurs during apoptosis of primary T lymphocytes (Ferraro-Peyret et al., 2002; Mandal et al., 2005).

The appearance of phosphotidylserine on the outer leaflet of apoptotic cells then facilitates noninflammatory phagocytic recognition, allowing for their early uptake and disposal (Fadok et al., 2001). This process of early and efficient uptake with no release of cellular constituents, results in essentially no inflammatory response. Table 4 lists the major proteins in the execution pathway with common abbreviations and some of the alternate nomenclature used for each protein.

Physiologic Apoptosis

The role of apoptosis in normal physiology is as significant as that of its counterpart, mitosis. It demonstrates a complementary but opposite role to mitosis and cell proliferation in the regulation of various cell populations. It is estimated that to maintain homeostasis in the adult human body, around 10 billion cells are made each day just to balance those dying by apoptosis (Renehan et al., 2001). And that number can increase significantly when there is increased apoptosis during normal development and aging or during disease.

Apoptosis is critically important during various developmental processes. As examples, both the nervous system and the immune system arise through overproduction of cells. This initial overproduction is then followed by the death of those cells that fail to establish functional synaptic connections or productive antigen specificities, respectively (Nijhawan et al., 2000; Opferman and Korsmeyer, 2003).

Apoptosis is also necessary to rid the body of pathogen-invaded cells and is a vital component of wound healing in that it is involved in the removal of inflammatory cells and the evolution of granulation tissue into scar tissue (Greenhalgh, 1998). Dysregulation of apoptosis during wound healing can lead to pathologic forms of healing such as excessive scarring and fibrosis. Apoptosis is also needed to eliminate activated or auto-aggressive immune cells either during maturation in the central lymphoid organs (bone marrow and thymus) or in peripheral tissues (Osborne, 1996).

Additionally, apoptosis is central to remodeling in the adult, such as the follicular atresia of the postovulatory follicle and post-weaning mammary gland involution, to name a couple of examples (Tilly, 1991; Lund et al., 1996). Furthermore, as organisms grow older, some cells begin to deteriorate at a faster rate and are eliminated via apoptosis. One theory is that oxidative stress plays a primary role in the pathophysiology of age-induced apoptosis via accumulated free-radical damage to mitochondrial DNA (Harman, 1992; Ozawa, 1995). It is clear that apoptosis has to be tightly regulated since too little or too much cell death may lead to pathology, including developmental defects, autoimmune diseases, neurodegeneration, or cancer.

Pathologic Apoptosis

Abnormalities in cell death regulation can be a significant component of diseases such as cancer, autoimmune lymphoproliferative syndrome, AIDS, ischemia, and neurode-generative diseases such as Parkinson's disease, Alzheimer's disease, Huntington's disease, and Amyotrophic Lateral Sclerosis. Some conditions feature insufficient apoptosis whereas others feature excessive apoptosis.

Cancer is an example where the normal mechanisms of cell cycle regulation are dysfunctional, with either an overproliferation of cells and/or decreased removal of cells (King and Cidlowski, 1998). In fact, suppression of apoptosis during carcinogenesis is thought to play a central role in the development and progression of some cancers (Kerr et al., 1994). There are a variety of molecular mechanisms that tumor cells use to suppress apoptosis.

Tumor cells can acquire resistance to apoptosis by the expression of anti-apoptotic proteins such as Bcl-2 or by the down-regulation or mutation of pro-apoptotic proteins such as Bax. The expression of both Bcl-2 and Bax is regulated by the *p53* tumor suppressor gene (Miyashita, 1994). Certain forms of human B cell lymphoma have overexpression of Bcl-2, and this is one of the first and strongest lines of evidence that failure of cell death contributes to cancer (Vaux et al., 1988). Another method of apoptosis suppression in cancer involves evasion of immune surveillance (Smyth et al., 2001).

Certain immune cells (T cells and natural killer cells) normally destroy tumor cells via the perforin/granzyme B pathway or the death-receptor pathway. In order to evade immune destruction, some tumor cells will diminish the response of the death receptor pathway to FasL produced by T cells. This has been shown to occur in a variety of ways including down-regulation of the Fas receptor on tumor cells. Other mechanisms include expression of nonfunctioning Fas receptor, secretion of high levels of a soluble form of the Fas receptor that will sequester the Fas ligand or expression of Fas ligand on the surface of tumor cells (Cheng et al., 1994; Cefai et al., 2001; Elnemr et al., 2001). In fact, some tumor cells are capable of a Fas ligand-mediated "counterattack" that results in apoptotic depletion of activated tumor infiltrating lymphocytes (Koyama et al., 2001).

Alterations of various cell signaling pathways can result in dysregulation of apoptosis and lead to cancer. The *p53* tumor suppressor gene is a transcription factor that regulates the cell cycle and is the most widely mutated gene in human tumorigenesis (Wang and Harris, 1997). The critical role of *p53* is evident by the fact that it is mutated in over 50% of all human cancers. *p53* can activate DNA repair proteins when DNA has sustained damage, can hold the cell cycle at the G_1/S regulation point on DNA damage recognition, and can initiate apoptosis if the DNA damage proves to be irreparable (Pientenpol and Stewart, 2002). Tumorigenesis can occur if this system goes awry. If the *p53* gene is damaged, then tumor suppression is severely reduced. The *p53* gene can be damaged by radiation, various chemicals, and viruses such as the Human papillomavirus (HPV). People who inherit only one functional copy of this gene will most likely develop Li–Fraumeni syndrome, which is characterized by the development of tumors in early adulthood (Varley et al., 1997; Gu et al., 2001).

The ataxia telangiectasia-mutated gene (ATM) has also been shown to be involved in tumorigenesis via the ATM/*p53* signaling pathway (Kitagawa and Kastan, 2005). The ATM gene encodes a protein kinase that acts as a tumor suppressor. ATM activation, via ionizing radiation damage to DNA, stimulates DNA repair and blocks cell cycle progression. One mechanism through which this occurs is ATM dependent phosphorylation of *p53* (Kurz and Lees-Miller, 2004).

As mentioned previously, *p53* then signals growth arrest of the cell at a checkpoint to allow for DNA damage repair or can cause the cell to undergo apoptosis if the damage cannot be repaired. This system can also be inactivated by a number of mechanisms including somatic genetic/epigenetic alterations and expression of oncogenic viral proteins such as the HPV, leading to tumorigenesis (Bolt et al., 2005). Other cell signaling pathways can also be involved in tumor development. For example, upregulation of the phosphatidylinositol 3-kinase/AKT pathway in tumor cells renders them independent of survival signals. In addition to regulation of apoptosis, this pathway regulates other cellular processes, such as proliferation, growth, and cytoskeletal rearrangement (Vivanco and Sawyers, 2002).

In addition to cancer, too little apoptosis can also result in diseases such as autoimmune lymphoproliferative syndrome (ALPS) (Worth et al., 2006). This occurs when there is insufficient apoptosis of auto-aggressive T cells, resulting in multiple autoimmune diseases. An overproliferation of B cells occurs as well, resulting in excess immunoglobulin production, leading to autoimmunity. Some of the common diseases of ALPS include hemolytic anemia, immune-mediated thrombocytopenia, and autoimmune neutropenia. The different types of this condition are caused by different mutations. Type 1A results from a mutation in the death domain of the Fas receptor, Type 1B results from a mutation in Fas ligand and Type 2 results from a mutation in caspase-10, reducing its activity.

Excessive apoptosis may also be a feature of some conditions such as autoimmune diseases, neurodegenerative diseases, and ischemia-associated injury. Autoimmune deficiency syndrome (AIDS) is an example of an autoimmune disease that results from infection with the human immunodeficiency virus (HIV) (Li et al., 1995). This virus infects $CD4^+$ T cells by binding to the CD4 receptor. The virus is subsequently internalized into the T cell where the HIV Tat protein is thought to increase the expression of the Fas receptor, resulting in excessive apoptosis of T cells.

Alzheimer's disease is a neurodegenerative condition that is thought to be caused by mutations in certain proteins such as APP (amyloid precursor protein) and presenilins. Presenilins are thought to be involved in the processing of APP to amyloid β. This condition is associated with the deposition of amyloid β in extracellular deposits known as plaques and amyloid β is thought to be neurotoxic when found in aggregated plaque form. Amyloid β is thought to induce apoptosis by causing oxidative stress or by triggering increased Fas ligand expressions in neurons and glia. It may also activate microglia, which would result in TNFα secretion and activation of the TNF-R1, leading to apoptosis (Ethell and Buhler, 2003).

Excessive apoptosis is also thought to play an important role in various ischemia-associated injuries. One example is myocardial ischemia caused by an insufficient blood supply, leading to a decrease in oxygen delivery to, and subsequent death of, the cardiomyocytes. Although necrosis does occur, overexpression of BAX has been detected in ischemic myocardial tissue and therapy aimed at reducing apoptosis has shown some success in reducing the degree of tissue damage (Hochhauser et al., 2003). One hypothesis is that the damage produced by ischemia is capable of initiating apoptosis but if ischemia is prolonged, necrosis occurs. If energy production is restored, as with reperfusion, the apoptotic cascade that was initiated by ischemia may proceed (Freude et al., 2000). Although the extent to which apoptosis is involved in myocardial ischemia remains to be clarified, there is clear evidence that supports a role for this mode of cell death.

Inhibition of Apoptosis

There are many pathological conditions that feature excessive apoptosis (neurodegenerative diseases, AIDS, ischemia, etc.) and thus may benefit from artificially inhibiting apoptosis. As our understanding of the field evolves, the identification and exploitation of new targets