State-of-the-Art Review

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Apoptosis: A review of pro-apoptotic and anti-apoptotic pathways and dysregulation in disease

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Abstract

Objective – To review the human and veterinary literature on the biology of apoptosis in health and disease.
Data Sources – Data were examined from the human and veterinary literature identified through Pubmed and references listed in appropriate articles pertaining to apoptosis.
Human Data Synthesis – The role of apoptosis in health and disease is a rapidly growing area of research in human medicine. Apoptosis has been identified as a component of human autoimmune diseases, Alzheimer's disease, cancer, and sepsis.
Veterinary Data Synthesis – Research data available from the veterinary literature pertaining to apoptosis and its role in diseases of small animal species is still in its infancy. The majority of veterinary studies focus on oncologic therapy. Most of the basic science and human clinical research studies use human blood and tissue samples and murine models. The results from these studies may be applicable to small animal species.
Conclusions – Apoptosis is the complex physiologic process of programmed cell death. The pathophysiology of apoptosis and disease is only now being closely evaluated in human medicine. Knowledge of the physiologic mechanisms by which tissues regulate their size and composition is leading researchers to investigate the role of apoptosis in human diseases such as cancer, autoimmune disease and sepsis. Because it is a multifaceted process, apoptosis is difficult to target or manipulate therapeutically. Future studies may reveal methods to regulate or manipulate apoptosis and improve patient outcome.

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Introduction

All tissues must be able to tightly control cell numbers and tissue size and to protect themselves from rogue cells that threaten homeostasis. In the early 1970s, Kerr et al,[1] observed a single-cell-death phenomenon that occurred in the dying cells of healthy tissues, as well as in cells associated with teratogenesis, neoplasia, tumor regression, atrophy, and involution. The term apoptosis, from Greek origins (*apo* = for, *ptosis* = falling), was chosen to describe the cellular process of programmed cell death.[1–3] Apoptosis is a tightly regulated intracellular program in which cells destined to die activate enzymes that degrade the cell's DNA and nuclear and cytoplasmic proteins.[4] Programmed cell death eliminates unwanted cells or potentially reactive cell lines either before or after maturation. This process is vital to fetal and embryonic development and to tissue remodeling.[3] Cell populations that normally have a high rate of proliferation, such as the intestinal epithelium, depend upon apoptosis to maintain the necessary number of cells.[5] The number of activated immune cells must be controlled to contain the inflammatory response.[6,7] Hormone-dependent apoptosis occurs during estrus and causes prostatic atrophy after castration.[8]

Kerr et al[1] observed that apoptotic cells share many morphologic features distinct from those in necrotic cells. Cells undergoing apoptosis exhibit 1 or more of the following: cell shrinkage, chromatin condensation and nucleosomal fragmentation, and bubbling of the plasma membrane (blebbing). Biochemical features of apoptosis include DNA fragmentation, protein cleavage at specific locations, increased mitochondrial membrane permeability, and the appearance of phosphatidylserine on the cell membrane surface.[3,9] There is an increase in mitochondrial permeability leading to the release of pro-apoptotic proteins and subsequent formation of apoptotic bodies. The resulting membrane-

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bound apoptotic bodies are consumed by neighboring cells or by macrophages. Apoptosis is a single-cell event, and does not induce an inflammatory reaction.

Apoptosis must be distinguished from necrosis, which is also a form of cellular death. In contrast to apoptosis, necrosis is not a genetically programmed function, it affects groups of neighboring cells, and produces an inflammatory response.[10] The death of a cell by necrosis leads to the release of alarm signal molecules that stimulate 1 or more pattern-recognition receptors on macrophages, dendritic cells, and natural killer cells.[11] The presence of necrotic cells in a tissue is frequently interpreted by the immune system as dangerous and therefore acts as a signal to initiate an immune response.[11] Unlike apoptosis, with necrosis there is cellular swelling with loss of cell membrane integrity, organelle swelling, and lysosomal leakage. The degradation of DNA is random and lysed cells are ingested by macrophages.[12]

Whether a cell survives or dies by apoptosis is determined by the balance between pro-apoptotic (stress or death) signals and anti-apoptotic (mitogenic or survival) signals within and around the cell (see Tables 1 and 2).

Table 1: Pro-apoptotic mediators

Agent	Mechanism
Extrinsic pathway	
TRAIL	Ligand binds to TNF-α receptor
TNF-α	Binds to TNF-α receptor; breakdown of sphingomyelin to ceramide
FasL	Binds to Fas receptor; breakdown of sphingomyelin to ceramide
DISC	Activation of caspase-8,-10; recruits c-FLIP; cleaves tBid to increase MOMP
TRADD, FADD	Adaptor proteins; death domain grouping recruits procasapase-8, -10
TWEAK	Ligand that binds to pro-apoptotic receptor
NGF	Ligand that binds to pro-apoptotic receptor
Intrinsic mitochondrial pathway	
Bcl-2 family proteins Group II, III	
BH3-only	Mediates diverse death stimuli from environment and from within cell; inactivates Bcl-2, Bcl-xL, triggering Bax/Bak
Bim	Binds and inhibits Bcl-2; may inactivate Bax/Bak
Bmf	Binds and inhibits Bcl-2
Bik, Bad	Binds and inhibits Bcl-2
Bid	Cleaved by caspase-8 to form tBid, which causes conformational change in Bax to allow it to insert into mitochondrial membrane inducing channel formation; may also inactive Bcl-2
PUMA, NOXA	Activates Bax to increase MOMP
BH1, 2, 3 or multi-domain	
Bax, Bak	Mitochondrial damage or changes cause cytochrome *c* release and ER depletion of calcium and caspase-12 activation
Mitochondrial substances	
AIF	Induces caspase-independent chromatin condensation and DNA fragmentation
Endo G	Breaks up DNA; caspase-independent
Smac/DIABLO, HtrA2/Omi	Binds and neutralizes IAPs
Procaspases-2,-3,-9	Initiates caspase cascade
Cytochrome *c*	Can cause reduction in mitochondrial membrane potential; binds with procaspase-9 and Apaf-1 to form apoptosome
ER pathway	Dissociation of TRAF2 and activation of caspase-12; release of cytochrome *c*
Caspases (effector)	
-3, -6, -7	Cleave cell membrane proteins, nucleus and cytoplasm
Alternate substances	
Granzyme B	Activates effector and activator caspases
Ceramide	Accumulates on mitochondrial membrane inhibiting Bcl-2; causes cytochrome *c* release; activates caspase-9 and effector caspases; activates Bax; releases cathepsins
p53	Suppresses the transcription of Bcl-2; induces the manufacture of Bax, insulin growth factor binding protein-3; upregulates the Fas receptor
Cathepsin D	Activates procaspase-3,-9; cleavage of Bid
c-Abl tyrosine kinase	Release of cytochrome *c* from the mitochondria

TRAIL, tumor necrosis factor-related apoptosis inducing ligand; FasL, Fas ligand; DISC, death-inducing signaling complex; c-FLIP, FLICE-like inhibitory protein; MOMP, mitochondrial outer membrane permeability; FADD, Fas-associated death domain; TRADD, TNFR1-associated death domain; TWEAK, TNF-like WEAK inducer of apoptosis; NGF, nerve growth factor; Bcl-2, B-cell lymphoma 2; BH3, Bcl-homology-3; tBid, truncated Bid; Bax, Bcl-2-associated protein x; Bak, Bcl-2-associated protein k; Apaf-1, apoptotsis activating factor-1 – activates procaspase 9; AIF, apoptosis inducing factor; Endo G, endonuclease G; IAPs, inibitor of apoptosis proteins; Smac/DIABLO, second mitochondrial-derived activator of caspases – director inhibitor of apoptosis-binding protein with LOw pI; TRAF2, TNF receptor associated factor 2.

Table 2: Anti-apoptotic mediators

Agent	Mechanism
Intrinsic pathway	
Bcl-2 (Group I), Bcl-xL	Controls mitochondrial permeability Inhibits Bax, Bak, granzyme B, p53
Extrinsic pathway	
c-FLIP	Prevents caspase-8 binding to various death receptors
NF-κB	Upregulates anti-apoptotic mediators c-FLIP, IAPs; accelerates growth; activates anti-apoptotic gene regulator p65
Alternate pathways	
IAP	Mimics Bcl-2; inhibits caspase-9 activity
Survivin	Regulates cell cycle mitosis; inhibits caspases-3,-7
XIAP	Inhibits caspase-3,-7,-9; activates NF-κB
Cytokine receptors	
JAK, STAT	Cytokine receptor interaction induces survival gene production through activation of NF-κB
MAPK	Translocates to nucleus; induces genetic production of anti-apoptotic factors
PKR	Phosphorylation of the protein initiation factor 2 alpha and IκB kinase complex; delays apoptosis
CDKs, cyclins, CDK inhibitors	Controls cell cycle machinery

Bcl-2, B-cell lymphoma 2; Bcl-xL, Bcl-2-associated protein xL; Bax, Bcl-2-associated protein x; Bak, Bcl-2-associated protein k; c-FLIP, FLICE-like inhibitory protein; NF-κB, nuclear factor-κB; IκB, inhibitory-κB; IAPs, inibitor of apoptosis proteins; XIAP, X-linked inhibitor of apoptosis protein; JAK, Janus kinase; STAT, signal transducers and activators of transcription; MAPK, mitogen-activated protein kinase; PKR, protein kinase R; CDK, cyclin-dependent kinase.

Cell injury via oxygen deprivation, heat stress, chemical agents, radiation, infectious agents, genetic derangements, nutritional imbalances, immunologic reactions (eg, anaphylaxis), and other types of severe cell stress will initiate the pro-apoptotic pathways.[4] Dysregulation of apoptosis can affect the equilibrium between cell growth and cell death, resulting in organ dysfunction.

Apoptosis in health is a finely balanced process. Too much or too little apoptosis contributes to disease. Apoptosis of infected cells is part of the host's defense mechanism. Some viruses and bacteria, however, have developed the ability to inhibit the infected cell's apoptotic mechanisms and protect their environment.[13] Inhibition of apoptosis is linked to uncontrolled cell growth and the formation of many types of cancer. In humans, excessive apoptosis is linked to stroke and Alzheimer's disease.[14] The activation or restoration of apoptosis is emerging as a key strategy for treatment of cancer and other diseases.[15–20]

Our aim is to provide a basic review of the literature regarding the mechanisms and regulation of apoptosis. Extracellular ligand-directed or intracellular stress-induced stimuli can activate this highly regulated process. Caspases play a central role by initiating and executing the intracellular cascade of events that result in protein and nucleic acid cleavage, and ultimately, cell death. Many of the key apoptotic proteins have been identified, however there is still much to learn regarding the molecular mechanisms of action or activation of these proteins. Knowledge of the pro-apoptotic and anti-apoptotic cell pathways is important to understanding the mechanisms of many life-altering diseases in humans and animals and realizing the potential for novel therapeutics.

Pathways of Apoptosis

Apoptosis can be genetically encoded or can occur in response to cellular or external stimuli. There are 3 features that characterize apoptosis: protein cleavage or hydrolysis, breakdown of nuclear DNA, and recognition of the apoptotic cell by phagocytic cells.[4] The cleavage of proteins primarily occurs with the activation of a family of cysteine proteases called caspases (Cysteine ASPartate-Specific ProteASEs).[8] Caspases are synthesized in an inactive form and activated by specific initiation mechanisms.[10] Programmed cell death can also result from caspase-independent mechanisms triggered by cell membrane receptor-ligand binding or damage to cell organelles.[21–26]

Initiation of Caspase Cascades

There are 3 known pathways that initiate the activation of caspase cascades and the programmed death of a cell. The route utilized is dependent on the initial death signal, the cell type involved, and the balance between pro-apoptotic and anti-apoptotic signals.[10] One initiating path may lead to another with cross-talk between them possible. Two of the pathways, the death receptor (DR) (extrinsic) and mitochondrial (intrinsic), have been detailed in the literature.[2,14,27–29] The third is an

intrinsic pathway involving the endoplasmic reticulum (ER) and is the least understood.[30–34] This pathway is believed to be a pathologically relevant form of apoptosis occurring in response to cellular stress.[10,35]

Extrinsic (DR) pathway: The extrinsic pathway (see Figure 1) begins with pro-apoptotic receptors on the cell's surface activated by a pro-apoptotic molecule or ligands specific for that receptor. These cell DRs belong to the tumor necrosis factor (TNF) receptor superfamily, with the Fas receptor and TNFR1 as the most intensely studied members.[15] Fas is present on a variety of cell types including activated B cells and T cells.[36] Ligands that activate pro-apoptotic receptors include the Fas ligand (FasL) and TNF-α[37–42] (Table 1). FasL is expressed by a variety of cell types, including activated T cells and natural killer cells.[36] TNF-α is produced predominantly by activated monocyte/macrophages and lymphocytes.[26]

The intracellular portion of the DR is known as the death domain (DD). Once 3 or more DR-ligand complexes bunch, their DDs are brought into close proximity and a binding site for an adaptor protein is formed. The adaptor protein is specific for that receptor (eg, Fas-associated DD [FADD] or TNF receptor-associated DD [TRADD]). This complex of ligand-receptor-adaptor protein is called the death-inducing signaling complex (DISC), leading to the recruitment and assembly of initiator caspases 8 and 10.[43–46] These caspases can now undergo self-processing and release active caspase enzyme molecules into the cytosol. Here, they activate the effector caspases 3, 6, and 7.[15,47,48] Figure 1 illustrates the sequence of events that trigger the extrinsic pathway.

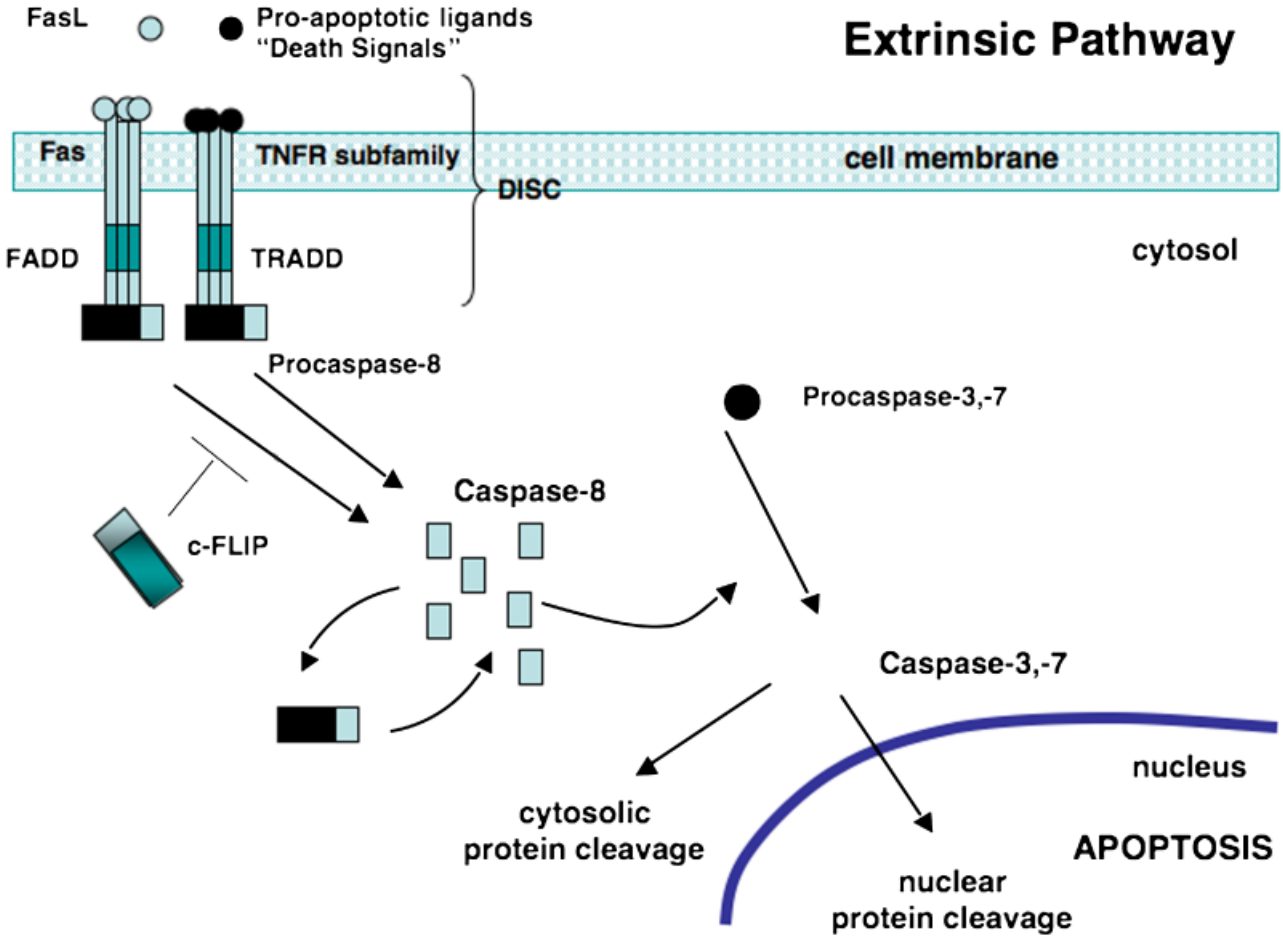

Figure 1: The extrinsic or death receptor (DR) pathway. Pro-apoptotic ligands, death signals, and Fas bind to Fas or TNFRs. The intracellular portion of the DR is known as the death domain (DD). Bunching of the receptor-ligand complexes groups their DDs and a binding site for an adaptor protein is formed. This ligand-receptor-adaptor protein complex is called the death-inducing signaling complex (DISC). It recruits and assembles initiator caspase-8 that releases active caspase enzyme molecules into the cytosol. Here, they activate the effector caspases-3 and -7, resulting in nuclear protein cleavage and the initiation of apoptosis. FasL, Fas ligand; TNFR tumor necrosis factor receptor; FADD, Fas-associated death domain; TRADD, TNF-associated death domain; c-FLIP, FLICE-like inhibitory protein; DISC, death-inducing signaling complex.

Intrinsic mitochondrial pathway: The intrinsic mitochondrial pathway (see Figure 2) is initiated from within the cell in response to cellular stresses such as DNA damage, radical oxygen species, radiation, hormone or growth-factor deprivation, chemotherapeutic agents, cytokines, and glucocorticoids.[30] Initiation of this pathway eventually results in the release of pro-apoptotic proteins from the mitochondria that will activate caspase enzymes and trigger apoptosis. [49–52] The success of the pathway in inducing apoptosis depends on the balance of activity between pro-apoptotic and anti-apoptotic members of the B-cell lymphoma-2 (Bcl-2) superfamily of proteins (Table 1).

Bcl-2 superfamily of proteins derives its name as the second member of a range of proteins found in follicular lymphoma.[4,53] All of the Bcl-2 family members are present on the outer mitochondrial membranes as dimers where they control membrane permeability in ion channel fashion or through the creation of pores.[53] The permeability of the mitochondrial outer membrane determines whether or not there is release of the pro-apoptogenic substances from the mitochondria.

This Bcl-2 family of proteins is subdivided into 3 groups based on structural similarities and functional criteria. Group I possess anti-apoptotic activity while groups II and III promote cell death.[2] The Bcl-2 family

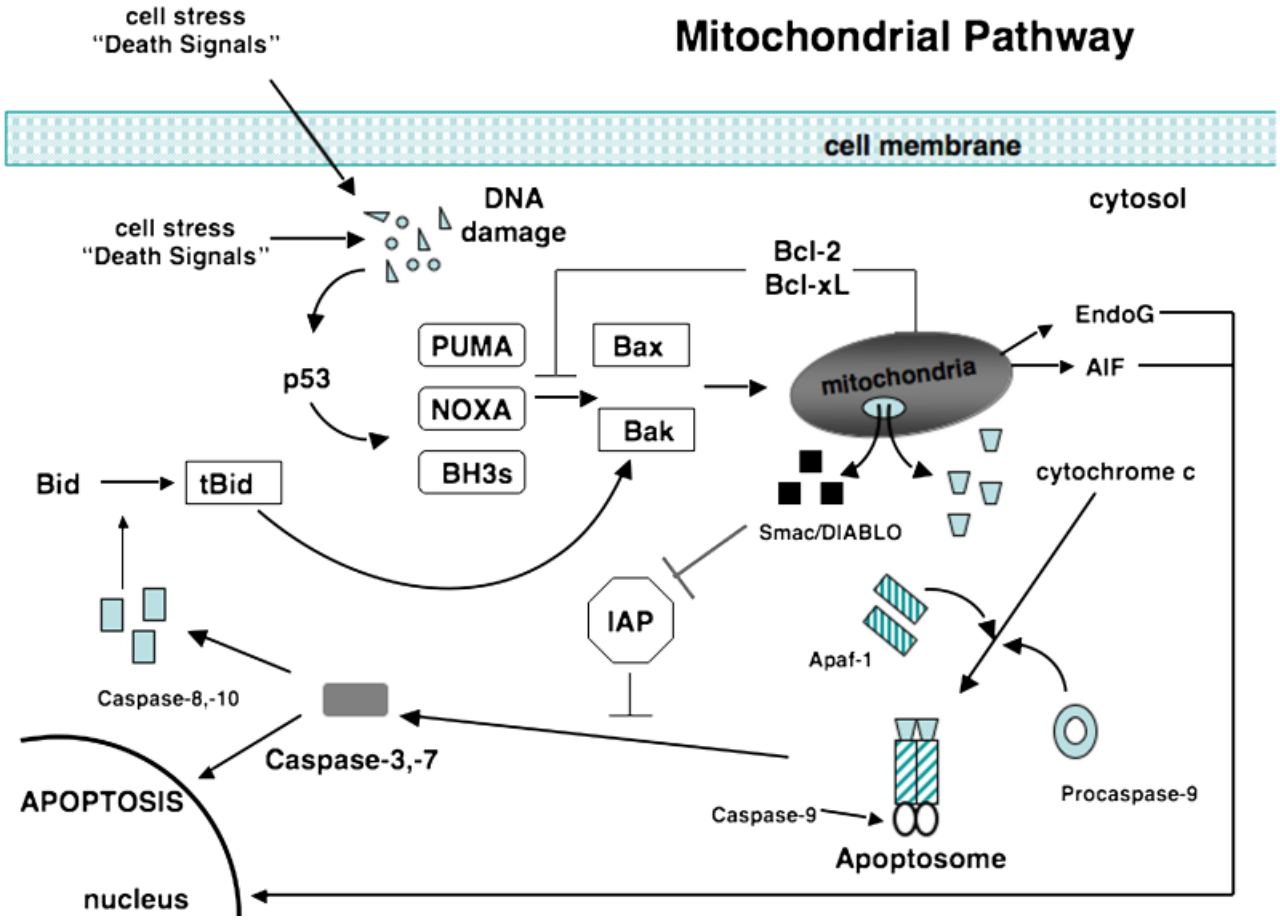

Figure 2: The mitochondrial or intrinsic pathway. Activation of the pro-apoptotic proteins Bax and Bak occurs through conversion of Bid to tBid by caspase-8 or-10 and through activation of PUMA, Noxa, or other BH3 initiator proteins when p53 is induced by DNA damage. Activated Bax and Bak oligomerize at the mitochondrial membrane and cause the release of several mitochondrial factors. Cytochrome *c* combines with Apaf-1 and procaspase-9 forming an apoptosome. Also released from the mitochondria are Smac/DIABLO, proteins that inactivate IAPs. Activated caspase-9 then is able to activate caspase-3 or -7 allowing apoptosis to proceed. Also released from the mitochondria are EndoG and AIF that stimulate apoptosis independent of caspases. Bcl-2 and Bcl-xL block the activation of Bax and Bak. Bcl-2, B-cell lymphoma-2; IAP, inhibitor of apoptosis protein; Apaf-1, apoptosis-activating factor-1; Smac, second mitochondrial-derived activator of caspases; DIABLO, director inhibitor of apoptosis-binding protein with LOw pI; BH3, Bcl-homology-3; tBid, truncated Bid; EndoG, endonuclease G; AIF, apoptosis-inducing factor; Bax, Bcl-2-associated protein x; Bak, Bcl-2-associated protein k.

share 1 or more of 4 characteristic domains of homology crucial for function. The anti-apoptotic Bcl-2 family proteins, such as Bcl-2 and Bcl-xL, contain all 4 domains and exert their control of mitochondrial permeability by stimulating ADP/ATP exchange, stabilizing the mitochondrial inner transmembrane potential, and preventing the opening of a permeability transition pore.[54] Overexpression of Bcl-2 and Bcl-xL is known to be associated with a number of human malignancies[49,55,56] (Table 2). These proteins also act by inhibiting the action of the pro-apoptotic proteins, Bax and Bak.

Pro-apoptotic proteins of the Bcl-2 family initiate apoptosis by blocking the anti-apoptotic activity of Bcl-2 and Bcl-xL by binding to their mitochondrial binding sites or by triggering the activation of pro-apoptotic Bax/Bak.[57] A third type of pro-apoptotic activity is through the cytoplasmic protein, Bid. This molecule is found in the cytoplasm in an inactive form. When cleaved by activated caspase-8 from the extrinsic pathway, Bid (once activated, referred to as t-Bid) causes a structural change to Bax making it similar to the structure of the anti-apoptotic molecule, Bcl-2, allowing Bax to translocate to the mitochondria.[58–61] This is but one method of cross-talk that occurs between the intrinsic and extrinsic pathways.

Each Bcl-2 family member can interact with other Bcl-2 members, so that large numbers of heterodimer combinations within a cell are possible. Cells with more pro-death proteins are sensitive to death and cells with an excess of protective family members are usually apoptosis-resistant.[2]

There are at least 3 current theories describing the exact mechanism by which the Bcl-2 pro-apoptotic proteins lead to increased mitochondrial permeability. The first theory describes the insertion of Bcl-2 proteins into the mitochondrial membrane and directly forming a channel.[2,62] A second theory explains the Bcl-2 pro-apoptotic proteins interacting with other mitochondrial membrane proteins, possibly voltage-dependent anion channel, to form large pores.[63] The size of the voltage-dependent anion channel is too small for proteins to pass, so this model assumes that there is a conformation change with Bcl-2 binding.[2] The third theory describes the Bcl-2 proteins modulating the mitochondrial proteins resulting in the formation of a permeability transition pore and loss of membrane potential, organelle swelling, and loss of cytochrome *c* from the pore.[64,65] Once the permeability of the membrane has been compromised, cytochrome *c* is released and combines with a cytosolic molecule called apoptosis activating factor-1 (Apaf-1). Cytochrome *c* and Apaf-1 combine with procaspase-9 for activation of this caspase (Figure 2). The binding of these 3 substances forms an apoptosome, which then activates procaspase-3.

Alternate substances can initiate the intrinsic and extrinsic pathways (Table 1). Phosphoprotein p53 is a transcription factor that regulates the cell cycle and functions as a cell stress sensor molecule capable of inducing the intrinsic mitochondrial pathway. Factors that damage DNA, such as ionizing radiation, genotoxic drugs, and free radicals, activate p53. Activated p53 promotes apoptosis primarily through its ability to suppress the transcription of anti-apoptotic factors like Bcl-2 or induce the manufacture of pro-apoptotic factors like Bax, insulin growth factor binding protein-3 and upregulation of the Fas receptor.[10,66] Bcl-xL can inhibit p53,[64] and inactivation or loss of p53 is a common abnormality of many human cancers.[66,67] One veterinary study showed an increased risk in cats, diagnosed with oral squamous cell carcinoma and exposed to secondhand tobacco smoke, to overexpress p53. In human cancer, p53 is the most commonly disrupted gene and is also the most frequently mutated gene in human oral cancer.[68]

Intrinsic ER pathway: The third and least understood pathway is referred to as the endoplasmic reticulum or ER pathway.[30] It involves caspase-12 and is said to be able to function independently of the mitochondria.[32] Cellular stresses such as hypoxia, glucose starvation, disturbances in calcium homeostasis, and exposure to free radicals injure the ER, resulting in the unfolding of proteins and reduction in protein synthesis. In normal cells, an adaptor protein, TNF receptor-associated factor 2 (TRAF2), is bound to procaspase-12, rendering it inactive. Stress of the ER leads to the dissociation of TRAF2, activation of caspase-12.[34,35,69] Once activated, caspase-12 cleaves procaspase-9, which then cleaves procaspase-3. This mechanism is independent of the mitochondria although there is evidence that caspase 12 can cause the release of cytochrome *c* from the mitochondria and thus stimulate the intrinsic pathway[35] (Table 1).

There are other mechanisms of cross-talk between the intrinsic pathways that initiate apoptosis. Ito et al,[69] demonstrated that ER stress can cause activation of a positive apoptosis regulator, c-Abl tyrosine kinase, known for its tumorogenic characteristics and results in the release of cytochrome *c* from the mitochondria.[25,69] In addition, the mitochondrial pathway pro-apoptotic Bcl-2 family protein, Bak, has been implicated in causing ER depletion of calcium, which can induce caspase-12 activation[70] (Table 1).

Caspase cascade

The central executioners of apoptosis are part of a large protein family known as the caspases. [2,71] To date, 14 caspases have been identified. Specific caspases are

found in relatively large amounts as inactive precursors called procaspases within the cytoplasm. Procaspases can be activated by 1 of 3 methods: (1) exposure to another activated caspase, (2) autocatalysis, or (3) association with an activator protein, such as caspase-9, Apaf-1, and cytochrome *c*.[2]

The caspases involved in apoptosis are subdivided into initiator caspases (2, 8, 9, 10) and effector (or executioner) caspases (3, 6, 7). The initiator caspases are activated by adaptor-mediated self-cleavage. The specific interaction of caspases and activator protein promotes the formation of a multimeric complex that is necessary to bring 2 caspase precursors together to activate each other and produce an active tetramer.[72,73] This caspase cascade strategy of activation is used by the initiator caspases to cleave and activate the effector caspases.[65]

When activated, the effector caspases selectively cleave a restricted set of target proteins that follow an aspartate residue. In most cases, this results in inactivation of the target protein. However, they can also activate proteins, either directly by cleaving off a negative regulatory domain, or indirectly, by inactivating a regulatory subunit.[2,74] Effector caspases cause cytoskeletal filament aggregation, clumping of ribosomal particles and rearrangement of rough ER to form a series of concentric whorls as seen on electron microscopy (Table 1).[10,65] Caspases are also responsible for cleaving nuclear lamins required for nuclear shrinking and budding, and for loss of cellular shape and membrane blebbing.[2,75,76]

Caspases will activate caspase-activated DNAases (CAD) that breakdown nuclear DNA, the second feature of apoptosis. CAD exists in an inactive form (ICAD) in the nucleus when it is bound to a subunit. Once the effector caspase-3 is activated it migrates to the nucleus and cleaves the inhibitory subunit thus activating CAD.[77] CAD is the nuclease responsible for breaking down the DNA into 50–300-kb pieces that are then cleaved into 180–200-bp fragments by endonucleases. It is these fragments that compose the DNA ladders visualized by agarose gel electrophoresis, a biochemical hallmark of apoptosis.[10]

Alternate pathways

Granzyme B: There is an accessory method of triggering apoptosis by the serine protease granzyme B, a lymphocyte granular enzyme expressed by activated cytotoxic T lymphocytes and natural killer cells. Granzyme B will bind to its cell surface receptor, an insulin-like growth factor II receptor, causing endocytosis of the protease. It remains in the endocytic vesicle until stimulation by an activated cytotoxic T cell.[78] Activation of almost all of the activator and effector caspases can occur through the action of the granzyme B pathway. A number of caspase sensitive targets, as well as other unique proteins not normally cleaved by caspases, can be cleaved directly by granzyme B.[74,79] This mechanism is dependent on mitochondrial disruption, as overexpression of anti-apoptotic Bcl-2 will halt this process. Granzyme-mediated apoptosis is integral to the immune surveillance machinery that rid the body of virally infected or malignantly transformed cells[26] (Table 1).

Mitochondrial factors: Increased mitochondrial outer membrane permeability can result in the release of mitochondrial pro-death substances in addition to cytochrome *c*, such as apoptosis inducing factor,[67] Smac/DIABLO[80–82] (Second Mitochondrial-derived Activator of Caspases/Direct Inhibitor of Apoptosis-Binding protein with LOw pI), endonuclease G, HtrA2/Omi, and several procaspases[83] (eg, procaspase-2, -3, and -9). Smac/DIABLO and HtrA2/Omi bind to cytoplasmic inhibitor of apoptosis proteins (IAPs), neutralizing their anti-apoptotic activity. The migration of apoptosis inducing factor from the mitochondria to the nucleus induces caspase-independent chromatin condensation and DNA fragmentation. Endonuclease G can also directly cause the break up of DNA independent of caspases[84] (Table 1, Figure 2).

Ceramide: The ceramide/sphingomyelin pathway can proceed with or without caspase interaction. Ceramides are sphingolipid-signaling mediators involved in the regulation of differentiation, growth suppression, cell aging, stress responses, and apoptosis. Various stresses, such as ultraviolet radiation, radical oxygen species, chemotherapeutics, IL-1β, TNF-α, or Fas activation, initiate this pathway.[21] Sphingomyelinases are activated by binding to TNF receptor family DD, FADD, and TRADD and cleave sphingomyelin, a member of the phospholipid bilayer, into ceramide.[22] Ceramide can also be generated on lysosomes, ER, and mitochondrial membranes, when stimulated by a variety of cytotoxic agents.[85–87]

Increased levels of ceramide initiate an apoptotic program involving mitochondrial membrane disruption (Table 1). Ceramide-induced apoptosis is mediated through the mitochondria when ceramide accumulates in the mitochondrial membrane. Elevation of ceramide and sphingosine result in increased mitochondrial membrane permeability, cyctochrome *c* release, and activation of caspase-9.[26] The susceptibility of a cell for ceramide-induced apoptosis is reliant on the cell's Bax to Bcl-2 ratio.[23] Ceramide production, DNA damage, and radical oxygen species also stimulate cellular lysosomal release of cathepsins, which leads to release of mitochondrial factors, activation of procaspase-9 and -3, and activation of Bid.[24,25]

Inhibitors of apoptosis

The most well-known anti-apoptotic factors are members of the Bcl-2 family (Table 2). There is a dynamic equilibrium between the anti-apoptotic members and pro-apoptotic members of the Bcl-2 family. Additional inhibitors include IAPs, FLICE-like inhibitory protein (c-FLIP) and nuclear factor-κB (NF-κB).

IAPs: A failsafe inhibitory mechanism exists in the intrinsic pathway. Pro-apoptotic activity is counterbalanced by a family of at least 8 proteins, known as IAPs (eg, survivin and XIAP). The IAPs compromise the effector phase of apoptosis through blocking or inactivating caspases[88–93] (see Table 2). They have been found associated with the activated TNF receptors[88] where they block the activation of caspase-8 and are upregulated by NF-κB activation.[89,90]

IAPs also act downstream of the mitochondrial release of cytochrome *c* to prevent activation of caspase-9.[94,95] Caspase-9 uses a peptide from one of its end subunits to attract an IAP family molecule. This binding allows caspase-9 to remain dormant even though the initial steps for activation have taken place.[96,97] The overexpression of IAPs is associated with a drug-resistant phenotype of cancer cells.[98]

c-FLIP: c-FLIP is a protein-deficient caspase homolog. This inhibitor prevents both the binding of caspase-8 to various DRs and its activation.[99] The ratio of c-FLIP to caspase-8 is critical for the assembly of the DISC. Formation of the DISC recruits c-FLIP to bind to its target molecule.[57] This works as a built-in safety system and alludes to the integral balance between pro-apoptotic and anti-apoptotic mediators.

Upregulation of c-FLIP has been associated with diverse hematologic cancer cell lines.[100,101] The sensitization of many cancer cells to death ligand-mediated apoptosis appears to be mediated by c-FLIP downregulation.[102,103] There does not appear to be inactivation of caspase-9 mechanisms because c-FLIP does not prevent apoptosis induced by granzyme B or by chemotherapeutic drugs and irradiation.[104]

NF-κB: NF-κB is a transcription factor and an anti-apoptotic gene regulator composed of a p50/p65 heterodimer. The p65 subunit provides the gene regulatory function. NF-κB is kept quiescent in the cytoplasm as a dimer bound to its repressor, inhibitors of NF-κB (IκB) family. Phosphorylation of IκB by upstream kinases frees NF-κB, which then translocates to the nucleus.[105] The p65 subunit is eventually released from the DNA and binds to newly synthesized IκB α, which complexes with NF-κB. This complex translocates back into the cytoplasm. Stimulation of NF-κB activation has been associated with accelerated growth, resistance to apoptosis, and propensity to form metastases. Inversely, inhibition of NF-κB activation produces an increase in apoptosis, indicating that the balance of cell viability versus cell death is maintained in some degree to NF-κB activation.[106]

Recognition of apoptotic cells

The third characteristic of apoptosis is recognition of the dying cells by phagocytic cells. The apoptotic cell expresses markers on their membrane that are recognized by phagocytes.[4] Through internal cellular signals phosphatidylserine, a phospholipid component, is shifted from the inner to the outer layer of the plasma membrane.[10] This allows for early recognition and removal of a dying cell without release of pro-inflammatory mediators, as occurs with necrosis.[4]

Regulation of apoptosis

Control of whether the pro-apoptotic or anti-apoptotic pathway is chosen is subject to positive and negative genetic and environmental regulators. Pro-apoptotic gene activation will lead to cell death while deactivation of the gene will block apoptotic pathways. Genetic regulation can also be modified by exogenous stimuli from the cell's immediate environment. A cell destined or started on a death pathway can receive a survival signal that can save the cell from apoptosis. Genetic regulators (mostly pro-apoptotic) include the c-Myc gene family, the p53 tumor suppressor gene, DRs and the caspase family.

The c-Myc gene is a member of the Janus kinase or JAK family, and is involved in both cell proliferation and apoptosis. In the presence of anti-apoptotic cytokines (eg, insulin-like growth factor-1) or negative regulators of apoptosis (eg Bcl-2), c-Myc drives cellular proliferation. In the absence of these factors, the c-Myc family promotes apoptosis.[10]

Survival signals, such as growth factors and other soluble mediators, are often released by neighboring cells. Hematopoietic cell lines and differentiated cells are dependent on survival factors like granulocyte macrophage colony stimulating factor, granulocyte colony stimulating factor, IL-3, and erythropoietin.[10] T and B lymphocytes are dependent on certain interleukins, such as IL-7 and IL-2, to mature properly or lead to cellular differentiation.[57] The role of environmental survival substances leads to the possibility of dysregulation of apoptosis in the presence of inappropriate survival signals, as observed in tumors and sepsis.[12,107,108]

Apoptosis in disease

Dysregulated apoptosis best describes pathologic disease states that induce or inhibit cell death inappropri-

ately. In humans, excessive apoptosis is linked to stroke and Alzheimer's disease and reduced apoptosis to cancer and autoimmune disease.[14,28,109] Apoptosis also plays a key role in sepsis. A review of the current literature in companion animal medicine yields research pertaining to apoptosis in the fields of oncology, orthopedics, and virology, as well as in other disease states. Table 3 lists some of the articles of veterinary origin that investigate apoptosis in disease.

Apoptosis in Cancer

A feature characteristic of cancer cells is the uncoupling of cell division and cell death; cells that should have died were not properly signaled to do so.[3] Oncologic studies are investigating the enhancement of apoptosis to arrest the growth of tumor cells. One mechanism through which normal cellular numbers are maintained is through p53 and its control over-apoptosis. Deficiencies in p53 can lead to reduced apoptosis and tumor development.[66,110] Some cancers harbor mutations in the p53 genome or disrupt normal p53 functions whereas others increase or overexpress Bcl-2 proteins leading to a cessation of the normal cellular death program.[78]

Apoptosis in sepsis

Cellular demise in sepsis can occur through apoptosis as well as necrosis. In 1996, Bone first proposed that apoptosis contributes to the multiple organ dysfunction in sepsis.[10,111] Most treatments had been aimed at blunting the over-reactive or pro-inflammatory response. Bone proposed that the anergic or hypoimmune aspect of sepsis, when apoptosis becomes most critical, must also be addressed,[111] Apoptotic loss of B cells, T cells, and dendritic cells in sepsis decreases antibody production, macrophage activation, and antigen presentation, respectively.[112]

Leukocytes are responsible for opsonization and phagocytosis of infected cells and antigens at the site of inflammation. Neutrophils produce highly toxic and unstable reactive oxygen species and release bactericidal substances. A hallmark of sepsis is the loss of normal apoptosis of neutrophils. This prolonged life produces neighboring cell damage and contributes to activation of pro-inflammatory cytokines. Normally, pro-inflammatory mediators (TNF-α, IL-1β, IL-6, IL-8, and IFN-γ) released from macrophages and neutrophils have overlapping effects and function to limit damage, combat pathogenic organisms, eliminate foreign antigens, and promote repair.[113] Anti-inflammatory cytokines (IL-4, IL-10, TGF-β, soluble receptors and receptor antagonists) are also quickly released to try to reduce and locally contain the inflammatory response.[114]Many of these inflammatory components are the key factors responsible for the dysregulated apoptosis of immune cells in sepsis.

Key cells involved in the inflammatory process (neutrophils, macrophages, dendritic cells, and lympho-

Table 3: Veterinary studies pertaining to apoptosis from 2005 to March 2008

Author	Journal	Topic
Oncology		
1. Scase, TJ et al	J Vet Intern Med 2006:20(1):151–8	Canine mast cell tumors and markers of apoptosis and cell proliferation
2. Johnson, ME et al	Vet Pathol 2004;41(6):599–607	Review of survivin, an IAP, and its role in cancer
3. Greissmayr, PC et al	J Vet Intern Med 2007;21(6):1409–12	Mushroom-derived Maitake PET fraction in the treatment of canine LSA
4. Fan, TM	Vet Clin North Am Small Anim Pract 2007;37(6):1091–110	Biphosphonates and cancer management
5. Lee, JJ et al	Am J Vet Res 2007;68(4):411–22	VP3 gene of chicken anemia virus causing apoptosis in K9 mammary tumor cells
6. Kano, R et al	Vet Clin Pathol 2008;37(1):57–60	Bcl-2 expression in feline lymphoma cell lines
7. Sano, J et al	Res Vet Sci 2005;79(3):197–201	Antineoplastic drug effects on Bcl-2 and Bcl-xL genes in feline T-cell leukemia
Orthopedics		
8. Lim, S et al	Vet Res Commun 2008;32(3):243–53	Enrofloxacin causes apoptosis in canine tendons and chondrocytes
9. Gyger, O et al	Vet J 2007;174(2):371–7	Apoptosis in normal and diseased canine cruciate ligaments
10. Echigo, R et al	J Vet Med Sci 2006;68(8):899–902	Hyaluronan decreases chondrocyte apoptosis in dogs with ACL injury
Viral		
11. Ruggieri, A et al	Vet Microbiol 2007;121(1–2):64–72	Canine coronavirus induces apoptosis through caspase-3 activation
12. Takano, T et al	Vet Microbiol 2007;119(2–4):121–31	TNF-α produced by FIP-infected macrophages induces and apoptosis in uninfected T cells
13. Natoni, A et al	J Gen Virol 2006;87(Pt 2):357–61	Mitochondrial pathway triggered during feline calicivirus infection
Miscellaneous		
14. Reusch, CE et al	Vet Rec 2007;160(7):219–24	Trilostane causing adrenal gland apoptosis and necrosis
15. Ammersbach, MA et al	J Vet Intern Med 2006;20(5):1166–71	Glucocorticoid immunosuppressive effects on canine lymphocytes may involve apoptosis

cytes) are also cells targeted for apoptosis. Apoptosis of immune cells is normally not a pathologic process because inflammatory cells must be eliminated so that inflammation does not continue unabated.[115,116] However, in sepsis or other overwhelming inflammatory processes (like trauma[117] and severe burns) there is extensive cell death of lymphocytes[118] and dendritic cells and delayed cell death of neutrophils. This leads to a blunted immune response coinciding with increased cellular damage.

Neutrophils are one of the first cells to migrate to the site of inflammation with an average half life of 6–12 hours when unstimulated.[119,120] Once a neutrophil is released into circulation, its apoptotic program has been activated. Studies have shown that sepsis can shorten as well as prolong the life span of the activated neutrophils (early or delayed apoptosis, respectively).[121–124]

Early apoptosis of neutrophils dampens respiratory burst activity and may lessen secondary tissue injury.[125] Delayed apoptosis of neutrophils contributes to increased cellular damage, especially in the lung, liver, kidneys, and gastrointestinal tract. Acute respiratory distress syndrome (ARDS) is marked by significant pulmonary accumulation of neutrophils,[126,127] and is considered to be a direct effect of neutrophil-induced injury. Cells retrieved from the lungs of septic patients show reduced rates of neutrophil apoptosis with the degree of inhibition paralleling the severity of sepsis.[128,129] Additional studies have shown that increased apoptosis (via Fas/FasL-dependent mechanism) of pulmonary epithelial cells will lead to permeability changes characteristic of ARDS.[130,131]

The cytokines produced by activated neutrophils summon macrophages to the area of inflammation; cytokines are also produced by tissue macrophages in response to foreign invasion. Macrophages are antigen-presenting cells (APC) capable of engulfing foreign material, infected cells, and apoptotic cells through recognition of specific cell surface molecules. Dendritic cells, another type of APC, are viewed as the sentinels of the immune system.[132] Like macrophages, mature dendritic cells are able to activate lymphocytes through the presentation of antigen.

Lymphocytes need 2 signals to stimulate differentiation and initiation of the immune response. The first signal is the presentation of antigen thus accounting for the specificity of the response. The second signal involves costimulatory molecules on APCs or APC secretion of cytokines. The costimulatory molecules interact with specific T cell sites producing a pro-apoptotic or anti-apoptotic state. Failure of the appropriate second signal after interaction with an APC results in anergy or apoptosis of the lymphocyte.[133,134] Anergy is a state of unresponsiveness to antigen.[112] This is also the mechanism for self-tolerance.[135]

Immature dendritic cells are capable of ingesting apoptotic cells but this will render them incapable of maturing and stimulating T cells.[136] Macrophages and dendritic cells will secrete IL-10 after engulfing apoptotic cells. IL-10, considered an anti-inflammatory cytokine, selectively blocks the maturation of dendritic cells.[137] This has been shown to suppress the phagocytic activity as well as pro-inflammatory cytokine production of alveolar macrophages.[138] IL-6 is also secreted by dendritic cells after ingesting apoptotic cells, leading to autocrine blockage of maturation.[139] This lack of maturation leads to a tolerogenic state with no further stimulation of the immune system.

After ingesting apoptotic cells, dendritic cells can mature only when there are danger signals expressed by the apoptotic cell or when dendritic cells are engulfing an excessive number of apoptotic cells. This leads to secretion of pro-inflammatory cytokines IL-1β and TNF-α by these signaled dendritic cells.[137] Inadequate clearance of surplus apoptotic cells results in these cells becoming necrotic and inducing a pro-inflammatory response.[140]

Studies have demonstrated apoptosis of intestinal and splenic B cells, CD4 T cells and dendritic cells in sepsis.[141–143] Overwhelming infection should lead to massive clonal expansion of B and T lymphocytes[142] but instead there is significant loss of these cell lines in sepsis. Lack of stimulation by APC cells experiencing apoptosis leads to poor B cell and T cell stimulation. These unstimulated T cells are removed by apoptosis.

In as many as 30% of bacteremic patients who die from systemic inflammatory response syndrome or multiple organ dysfunction syndrome, no focus of infection is identified. Premature B cell and intestinal epithelial cell death through apoptosis in the intestines is one theory to explain intestinal bacterial translocation and loss of the first line of intestinal defense.[10]

Treatment strategies

Current human treatment strategies for manipulating apoptosis focus mainly on cancer and sepsis. A Pubmed literature search at the time of writing revealed a total of 645 citations for apoptosis and veterinary. Most of the studies are experimental at this point but many oncologic studies are manipulating apoptosis in the treatment of their patients (Table 3).

In human cancer research, peptidomimetics are in the early stages of experimental study. They are synthetic peptides that are resistant to enzymatic degradation and are being used for their pro-apoptotic effects on Bid as well as their antagonism of IAPs.[144,145] In cancer

treatments, there are trials attempting to block the overexpression of Bcl-2 because it is the cessation of normal apoptosis that leads to the growth of tumors. In addition to the direct effects on the apoptotic programs, discoveries are being made that allow chemotherapeutics to act synergistically with various anti-apoptotic therapeutics.[78]

Treatment strategies for sepsis had previously targeted the hyperimmune phase rather than the hypoimmune phase. Addressing apoptosis can be therapeutically challenging because targeted blockade of apoptosis in lymphocyte populations must be specific enough to primarily target those cell populations undergoing increased apoptosis and to be sufficiently transient to prevent the risk of malignant transformation associated with prolonged blockade of apoptosis.[146] This poses a challenge to try to target specific pathways. Attempts at blocking the circulating mediators and cytokines that induce apoptosis have not been successful because of the inherent redundancy and fail-safe mechanisms of the apoptotic pathways.[119] Caspase inhibitors show promise because caspases are common factors to many apoptotic pathways. Broad-spectrum caspase inhibitors have been shown to prevent lymphocyte apoptosis and improve survival in animal models of sepsis.[13,147,148] Caution must be expressed, though, because increased dosages of caspase inhibitors can cause cytotoxicity and TNF-α induced injury.[149,150]

Gene therapy studies have shown that overexpression of Bcl-2 delays or blocks apoptosis and improves survival in septic mice.[10,151,152] Other therapies target Akt, a regulator of cell proliferation and death. Mice overexpressing Akt have reduced lymphocyte apoptosis and increased survival after cecal ligation and puncture.[153] Fas fusion proteins, and attempts at altering gene expression of members of the DR pathways, are also areas of ongoing apoptotic research.[147,154–157] Recombinant human activated protein C, a product being used successfully in some septic patients,[158,159] may counteract the induction of apoptosis in monocyte[160] and endothelial cell lines, modulating the inflammatory and coagulation cascades during sepsis.[161–166] It also helps to attenuate the levels of pro- and anti-apoptotic proteins in favor of survival.

In contrast to anti-apoptotic strategies, recent studies have addressed the hypothesis that apoptosis in sepsis may in some cases be beneficial by downregulating the inflammatory response. Earlier onset of apoptosis may, in fact, favor survival. Giarmarellos-Bourboulis et al[167] have associated monocyte apoptosis at the onset of sepsis to a favorable outcome due to a decrease in the amount of pro-inflammatory cytokines produced.

Summary

Apoptosis is a normal biologic process necessary to maintain cellular homeostasis. There are characteristic pathways that lead to this form of cell death continually influenced by local cellular events, growth factors, and neighboring stresses. This complicated system has numerous built-in avenues and failsafe mechanisms, including pro-apoptotic and anti-apoptotic factors. During sepsis and cancer, just 2 of the many diseases causing dysregulation of the apoptotic process, cells are either killed too quickly or survive too long. Newer therapies, designed to manipulate apoptosis depending on the pathology involved, can promote or delay this form of cellular demise. Although an extremely complicated process, understanding the relationship between sepsis and apoptosis will undoubtedly lead to new treatment modalities.

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