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Review article

Apoptosis: from Signalling Pathways to Therapeutic Tools

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Abstract

Apoptosis or programmed cell death is a gene regulated phenomenon which is important in both physiological and pathological conditions. It is characterized by distinct morphological features including chromatin condensation, cell and nuclear shrinkage, membrane blebbing and oligonucleosomal DNA fragmentation. Although, two major apoptotic pathways including 1) the death receptor (extrinsic) and 2) mitochondrial (intrinsic) pathway have been identified, recently endoplasmic reticulum and lysosomal pathways have been also recognized. Depending on both the cell type and the initiating factor, distinct pathways are activated. The pathways share a common final phase of apoptosis, consisting of activation of the executioner caspases and dismantling of substrates critical for cell survival. The important regulatory mechanisms include death receptors, caspases, mitochondria and Bcl-2 family proteins. Modulating of apoptosis is a novel therapeutic strategy in treatment of different diseases. These include situations with unwanted cell accumulation (cancer) and failure to diminish aberrant cells (autoimmune diseases) or diseases with an inappropriate cell loss (heart failure, stroke, AIDS and neurodegenerative diseases). Modulation of apoptosis is a novel therapeutic strategy in treatment of different diseases. Many approaches including gene therapy, antisense strategies and numerous apoptotic drugs to target specific apoptotic regulators, are currently being developed. The goal of this review is to provide a general overview of current knowledge on the process of apoptosis including morphology, biochemistry, signaling as well as a discussion of apoptosis in diseases and effective therapy.

Keywords: Apoptosis, Autoimmunity, Cancer, Intrinsic/Extrinsic pathway, Neurodegenerative diseases

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Introduction

Apoptosis, or cell suicide, is a form of cell death that is morphologically and biochemically distinct from necrosis. The term apoptosis (a-po-toe-sis) was first used by Kerr in 1972 (1-3) Apoptosis is 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 (4-6). Apoptosis is a gene regulated phenomenon which is important in both physiologicalal and pathological conditions. It plays an important role during development, metamorphosis and in many diseases including autoimmune, neurodegenerative, cancer and AIDS (7-9). Apoptosis plays an important role during development, metamorphosis and in many diseases (10). Failure to regulate apoptosis is a common feature in several diseases (11). In case of cancers, triggering of apoptosis in malignant cells will be a way of disease control. Alternatively, in a pathological process which induces cellular degeneration, apoptosis inhibition will be expected to be helpful. Apoptosis also occurs as a defense mechanism such as in immune reactions or when cells are damaged by disease or noxious agents (12). Different injurious stimuli such as heat, radiation, hypoxia, reactive oxygen species (ROS) and cytotoxic anticancer drugs can induce apoptosis (13). Apoptosis-inducing compounds are good candidate in cancer chemotherapy.

Morphological features of apoptosis

Apoptosis is characterized by distinct morphological features including; chromatin condensation, cell and nuclear shrinkage, membrane blebbing and oligonucleosomal DNA fragmentation (14). During the early process of apoptosis, cell shrinkage and pyknosis are visible by light microscopy which cells are smaller in size, the cytoplasm is dense and the organelles are more tightly packed (1). Pyknosis is the result of chromatin condensation and this is the most characteristic feature of apoptosis. 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. 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. Apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue and there is no inflammatory reaction associated with apoptosis (15, 16).

Biochemical features of apoptosis

Apoptotic cells exhibit several biochemical modifications such as protein cleavage, protein cross-linking, DNA fargmentation, and phagocytic recognition that together result in the distinctive structural pathology described previously (17). The central hydrolytic reactions of apoptosis are catalyzed by a family of proteases, now termed "caspases" for "cysteine proteases acting on aspartic acid". At least 12 of these enzymes are known. 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. For example, when multiple procaspase-9 molecules assemble on the apoptosomes, they can cleave one another to remove a leader sequence and generate a short and long peptide (18). Caspases are often classified as initiators (caspase-2, 8, 9, 10), effectors or executioners (caspase-3, 6, 7) and inflammatory caspases (caspase-1, 4, 5) (19, 20). Effector caspases cleave and inactivate proteins that protect living cells from apoptosis, such as the DNA repair protein, poly (ADP-ribose) polymerase (PARP), ICAD/DFF45 (inhibitor of caspase-activated DNase, the nuclease responsible for DNA fragmentation), or the anti-apoptotic Bcl-2 proteins. Other actions of caspases in apoptosis include cleavage of cytoskeletal

proteins, including the lamins, proteins forming the nuclear lamina; cytoplasmic intermediate filaments (vimentin, cytokeratins), and several proteins involved in cytoskeleton regulation (gelsolin, focal adhesion kinase and p21-activated kinase 2). This results in disassembly of cell structures that depend on the cytoskeleton (18).

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 (21-24).

Extensive protein cross-linking is another characteristic of apoptotic cells and is achieved through the expression and activation of tissue transglutaminase (25). DNA breakdown by Ca^{2+}-and Mg^{2+}-dependent endonucleases also occurs, resulting in DNA fragments of 180 to 200 base pairs (26). 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, leading to quick phagocytosis. 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. Recent studies have shown that other proteins including Annexin I and calreticulin are also be exposed on the cell surface during apoptosis (27). 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. 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 (28). 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 (28-30).

Distinguishing apoptosis from necrosis

Necrosis is an uncontrolled, passive process and an energy-independent mode of death that usually affects large fields of cells whereas apoptosis is controlled and energy-dependent and can affect individual or clusters of cells (31, 32). Necrosis is mediated by two main mechanisms; interference with the energy supply of the cell and direct damage to cell membranes (33, 34). Some of the major morphological changes that occur with necrosis include cell swelling; formation of cytoplasmic vacuoles; distended endoplasmic reticulum; condensed, swollen or ruptured mitochondria; disaggregation and detachment of ribosomes; disrupted organelle membranes; swollen and ruptured lysosomes and eventually disruption of the cell membrane (1, 34, 35). 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 (15, 16). 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 (36). Table 1 compares some of the major morphological features of apoptosis and necrosis. 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 physiological milieu (32, 37). Two factors that will convert an ongoing apoptotic process into a necrotic process availability of caspases and intracellular ATP. 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 (38, 39).

Table 1. Comparison of morphological features of apoptosis and necrosis.

Apoptosis	Necrosis
Cell shrinkage and convolution	Cell swelling
Cytoplasm retained in apoptotic bodies	Cytoplasm released
Intact cell membrane	Disrupted cell membrane
No inflammation	Inflammation usually present

In addition to inducing apoptosis, a number of chemotherapeutic agents have been reported to induce non-apoptotic forms of cell death (40-42). For example, DNA alkylating agents kill cells resistant to apoptosis by inducing necrosis (42).In regard to melanoma, we demonstrated that Ingenol 3-angelate, one of the active ingredients in an extract from *Euphorbia peplus*, and rose bengal induce caspase-independent non-apoptotic cell death(43, 44). The significance of non-apoptotic forms of cell death in chemotherapy and the mechanism(s) by which they are induced by chemotherapeutic drugs remain, largely unclear. It is however noteworthy the non-apoptotic cell death is often observed under conditions in which apoptosis is inhibited (44).

Apoptotic pathways

To date, two major apoptotic pathways have been identified - the death receptor (extrinsic) and mitochondrial (intrinsic) pathway (45, 46). Although each pathway is initially mediated by different mechanisms, they share a common final phase of apoptosis, consisting of activation of the executioner caspases and dismantling of substrates critical for cell survival (47, 48). However, there is now evidence that these pathways are linked and that molecules in one pathway can influence the other (49) (Figure 1).

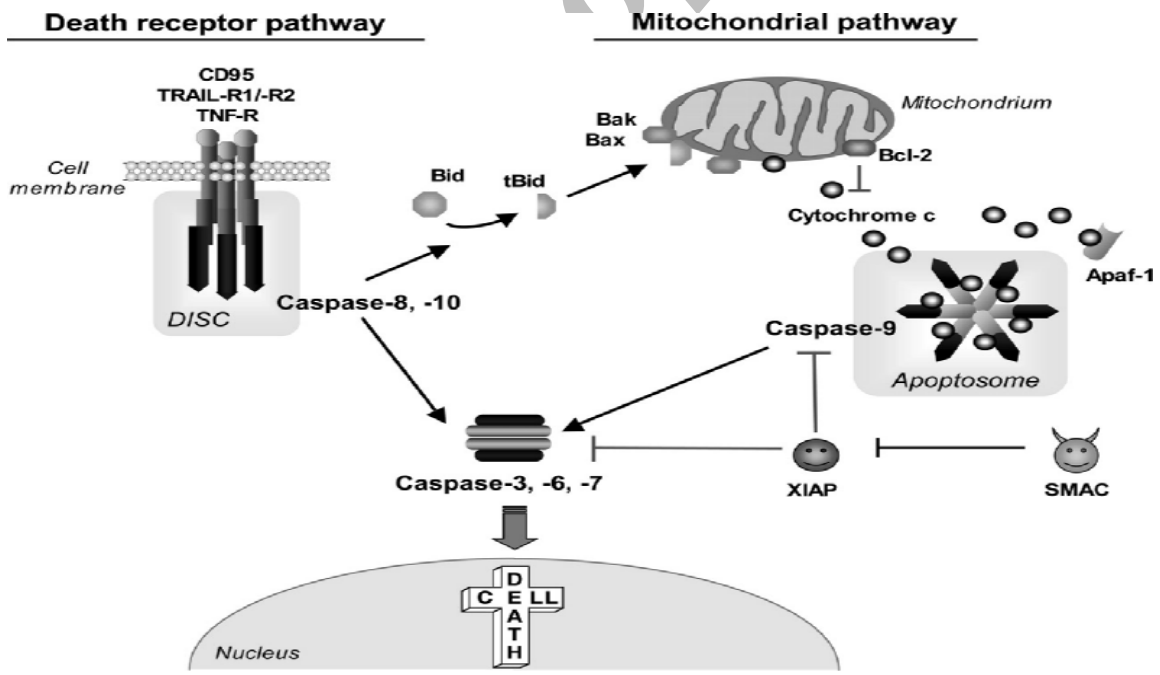

Figure 1. Two major pathways of apoptosis exist in mammalian cells. Left, the extrinsic cell death pathway is mediated by a subgroup of the TNF receptor superfamily called death receptors (CD95, TRAIL-R1/2, and TNF-R1). Receptor-mediated cell death results in the activation of caspase-8, which then directly cleaves and activates caspase-3, -6, or -7, the executioner enzymes of apoptosis.
Right, mitochondrial or intrinsic pathway, is initiated by multiple forms of cellular stress. Intrinsic pathway triggers the assembly of the apoptosome (Apaf-1 and caspase-9) and subsequent activation of caspase-3 and cell death. Pro-apoptotic Bcl-2 family members Bax and Bak translocate to the mitochondria. The BH3-only protein Bid activates Bax and Bak to mediate the release of cytochrome c in the cytosol. The inhibitory function of IAPs is countered by the SMAC (Adapted with permission) (50).

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 (51). 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" (45). 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 (Figure 1) (45, 52-55).

The sequence of events that define the extrinsic phase of apoptosis are best characterized with 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 (56, 57). 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 (58).

Once caspase-8 is activated, the execution phase of apoptosis is triggered. Death receptor ediated apoptosis can be inhibited by a protein called c-FLIP which will bind to FADD and caspase-8, rendering them ineffective (59, 60). 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 (61). Table 2 lists the major extrinsic pathway proteins with common abbreviations and some of the alternate nomenclature used for each protein.

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor family, such as the tumor necrosis factor α and Fas ligand, which is a type 2 membrane protein that can induce apoptotic cell death in a wide range of cultured malignant cells, but not normal tissues. Induction of apoptosis by TRAIL is believed to be mediated by its interaction with 2 death receptors on cells referred to as TRAIL-R1 (DR4) and TRAIL-R2 (DR5) (62). It is postulated that normal cells are protected from TRAIL-induced apoptosis by their expression of TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2), which lack cytoplasmic death domains and act to sequester TRAIL (decoy receptors, DcRs) or to mediate anti-apoptotic signals. (43, 62-74).

Mitochondrial 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.

Table 2. Some of proteins involved in extrinsic pathway (74, 75).

Abbreviation	Protein Name
Apo2L	Apo2 ligand
Apo3L	Apo3 ligand
Caspase 8	Cysteinyl aspartic acid-protease 8
DED	Death effector domain
DR3	Death receptor 3
DR4	Death receptor 4
DR5	Death receptor 5
FADD	Fas-associated death domain
FasL	Fatty acid synthetase ligand
FasR	Fatty acid synthetase receptor
RIP	Receptor-interacting protein
TNFR1	Tumor necrosis factor receptor 1
TNF-α	Tumor necrosis factor alpha
TRADD	TNF receptor-associated death domain

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 result 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 (77).

The first group consists of cytochrome C, Smac/DIABLO, and the serine protease HtrA2/Omi (78-80). These proteins activate the caspase-dependent mitochondrial pathway. Cytochrome C binds and activates Apaf-1 as well as procaspase-9, forming an "apoptosome" (81, 82).

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 (83, 84). 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" (85). Apoptosis induced by TRAIL in melanoma cell lines is also caspase-dependent (86).

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 (87). This early form of nuclear condensation is referred to as "stage I" condensation (88).

Endonuclease G also translocates to the nucleus where it cleaves nuclear chromatin to produce oligonucleosomal DNA fragments (89). 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 (90). This later and more pronounced chromatin condensation is referred to as "stage II" condensation (88). In our previous studies we also report that apoptoss induced by Staurosporine and rose bengal in melanoma cells is both caspase- dependent and in-dependent (13, 91).

Bcl-2 family proteins

The control and regulation of these apoptotic mitochondrial events occurs through members of the Bcl-2 family of proteins (92). 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 (93). 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.

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. In response to apoptotic stimuli, several pro-apoptotic proteins are translocated to the mitochondria, where they can interact with membranebound anti-apoptotic proteins, thereby inhibiting the survival functions of the latter (94). Bcl-2,

Bcl-XL, and Bax can form ion channels in artificial membranes, suggesting regulation of apoptosis via the formation of pores.56,61 Other hypotheses for the inhibition of apoptosis by Bcl-2 include participation in an anti-oxidant pathway62 and blockage of the release of cytochrome C. (94) In the absence of a death signal, pro-apoptotic Bcl-2 family members are often sequestered by cytoskeletal elements or cytoplasmic proteins (*e.g.*, sequestration of phosphorylated Bad by 14–3–3 proteins) or are only loosely associated with membranes. In contrast, anti-apoptotic Bcl-2 family members are often integral membrane proteins found in the mitochondrial membrane, the nuclear envelope, and the endoplasmic reticulum (94) The activity of Bcl-2-related proteins is regulated through several mechanisms, including their levels of expression, sequestration, and post-translational modifications, such as phosphorylation, cleavage, and translocation Mitochondrial damage in the Fas pathway of apoptosis is mediated by the caspase-8 cleavage of Bid (95, 96). This is one example of the "cross-talk" between the death-receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway (49). 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 (97).

Bad can also heterodimerize with Bcl-XL or Bcl-2, neutralizing their protective effect and promoting cell death (98). 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 (99). An additional protein designated "Aven" appears to bind both Bcl-XL and Apaf-1, thereby preventing activation of procaspase-9 (100). 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 Bcl-2 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 (101). 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 (102). 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 (103).

The ratio of pro- to anti-apoptotic members has been suggested to regulate cell life or death. In our studies increased Bax/Bcl-2 expression has been shown in glucose- and lead-induced apoptosis in PC12 cells (104, 105).

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.

Endoplasmic reticulum and Lysosomal pathways

It has become clear that each of the main cellular organelles including endoplasmic reticulum (ER) and lysosome can participate in cell death signaling pathways. Recent advances have highlighted the importance of the ER in cell death processes (106). The efficient functioning of the endoplasmic reticulum (ER) is essential for most cellular activities and survival.

Table 3. Some of proteins involved in intrinsic pathway (74).

Abbreviation	Protein Name
AIF	Apoptosis Inducing Factor
Apaf-1	Apoptotic protease activating factor
BAD	Bcl-2 antagonist of cell death
BAG	Bcl-2 associated athanogene
BAK	Bcl-2 antagonist killer 1
BAX	Bcl-2 associated X protein
Bcl-10	B-cell lymphoma protein 10
Bcl-2	B-cell lymphoma protein 2
Bcl-w	Bcl-2 like 2 protein
Bcl-x	Bcl-2 like 1
Bcl-XL	Bcl-2 related protein, long isoform
Bcl-XS	Bcl-2 related protein, short isoform
BID	BH3 interacting domain death agonist
BIK	Bcl-2 interacting killer
BIM	Bcl-2 interacting protein
Blk	Bik-like killer protein
CAD	Caspase-Activated DNAse
Caspase-9	Cysteinyl aspartic acid-protease-9
IAP	Inhibitor of Apoptosis Proteins
Myc	Oncogene Myc
Noxa	Phorbol-12-myristate-13-acetate-induced protein 1
Puma	Bcl-2 binding component 3
Smac/DIAB	Second mitochondrial activator of caspases/direct IAP binding protein with low

Conditions that interfere with ER function lead to the accumulation and aggregation of unfolded proteins (107). ER transmembrane receptors detect the onset of ER stress and initiate the unfolded protein response (UPR) to restore normal ER function. If the stress is prolonged, or the adaptive response fails, apoptotic cell death ensues (107, 108). Many studies have focused on how this failure initiates apoptosis, as ER stress-induced apoptosis is implicated in the pathophysiology of several neurodegenerative and cardiovascular diseases including alzheimer disease, parkinson disease, and type 2 diabetes (109-111). Recent work has shown that the Bcl-2 family of proteins plays a central role in regulating this form of cell death, both locally at the ER and from a distance at the mitochondrial membrane. The existence of Bcl-2-regulated initiator procaspase activation complexes at the ER membrane has also been described (110, 112). In addition to propagating death-inducing stress signals itself, the ER also contributes in a fundamental way to Fas-mediated apoptosis and to p53-dependent pathways resulting from DNA damage and oncogene expression. Mobilization of ER calcium stores can initiate the activation of cytoplasmic death pathways as well as sensitize mitochondria to direct pro-apoptotic stimuli (113).

Lysosomes may function as death signal integrators. Rupture of lysosomes, leading to the release of their cathepsin content, has long been recognized as potentially harmful to the cell (114). Strong evidence is now accumulating for the involvement of alternative proteases, such as cathepsin B (CB), in apoptosis (115), but the molecular identity of the mediators and the necessity of activation of the apoptotic pathways remain to be elucidated in most cases and may vary on the type of cells and the applied death stimulus (116). CB has been reported to contribute to apoptosis via induction of mitochondrial membrane permeabilization, possibly via cleavage of Bid, in some systems, thereby acting upstream of the caspase cascade (117-119). Recent evidence has suggested that cathepsin D is involved in apoptosis induced by a number of conventional anti-cancer agents, including etoposide, cisplatin and 5-fluorouracil. The mechanisms leading to release of cathepsin from the lysosomes after treatment with these agents are unclear as is the relative importance of the lysosomal pathway for the cytotoxicity of these compounds (108, 120-124).

Table 4. Some of proteins involved in execution phase (74, 75).

Abbreviation	Protein name
CAD	Caspase-activated DNAse
Caspase-10	Cysteinyl aspartic acid-protease-10
Caspase-3	Cysteinyl aspartic acid-protease-3
Caspase-6	Cysteinyl aspartic acid-protease-6
Caspase-7	Cysteinyl aspartic acid-protease-7
ICAD	Inhibitor of CAD
PARP	Poly (ADP-ribose) polymerase

Apoptosis and Pharmacotherapy

Modulating of apoptosis is a novel therapeutic strategy in treatment of different diseases. These include situations with unwanted cell accumulation (cancer) and failure to eradicate aberrant cells (autoimmune diseases) or disorders with an inappropriate loss of cells (heart failure, stroke, AIDS, neurodegenerative diseases, and liver injury). Many approaches including gene therapy, antisense strategies and numerous apoptotic drugs to target specific apoptotic regulators, are currently being developed (50).

Apoptosis and cancer

Defects in apoptosis play important roles in tumor pathogenesis, allowing neoplastic, as well as genetically unstable cells, to survive (125). Moreover, deregulation of apoptosis affects chemo- and radioresistance, increasing the threshold for cell death and facilitating metastasis (126-128). Apoptotic strategies to kill tumor cells can involve direct induction of pro-apoptotic molecules, modulation of anti-apoptotic proteins, or restoration of tumor suppressor gene functions. Death receptors have been pursued as potential targets for cancer therapy. Candidates such as TNF death receptor family have been investigated after observing promising anti-tumor activity *in vitro*. However, TNF was shown to be ineffective in triggering cancer cell killing in vivo, in addition to its toxic side effects. The problems seen with TNF and Fas were overcome when TRAIL (Apo2) emerged as potential anticancer agent. TRAIL and agonist antibodies against TRAIL are well tolerated *in vivo*. Indeed, a phase I trail has been recently completed in humans, raising the possibility of using these biological agents as a novel approach in cancer treatment (Table 1).

Other routes, such as protein kinase C (PKC) may be important in leukemia, as it has been demonstrated that some PKC modulators stimulate myeloid leukemia cell lines to produce TNF, resulting in apoptosis induction. A great part of the solid tumors overexpress growth factor receptors such as EGFR (epidermal growth factor receptor). Herceptin (Hoffmann-La Roche), an antibody blocking the EGF-R type 2 (Her2/neu), was one of the first rationally designed drugs that is now successfully applied in metastatic breast cancer (129). In addition, Gefitinib/Iressa (AstraZeneca) has been approved for treatment of non-small-cell lung cancer as a potent, selective ATP-competitive inhibitor of EGF-R tyrosine kinase, which inhibits growth of many different cell lines. As well as inhibiting tumor cell proliferation, Gefitinib treatment increases apoptosis (130, 131), reduces invasiveness (132, 133) and decreases angiogenesis in some tumor cells (134). The degradation and elimination of cells in apoptosis is dependent on the degradation of cellular proteins by caspases.

Active caspases have been engineered by fusing one or more chemically inducible dimerization domains. These engineered molecules are named artificial death switches. This synthetic activation of caspases has been shown to be effective in prostate cancer cell lines (135). Tumor cells may be preferentially sensitive to agents that trigger the lysosomal apoptosis pathway (136). The degree of lysosomal permeabilization may determine the amounts of cathepsins released into the cytosol: a complete breakdown of all lysosomes will result in necrosis, whereas partial breakdown may trigger apoptosis (137). Lysosomal cathepsins including cathepsins B, D, and L translocate from the lysosomal lumen to the cytosol in response to a variety of signals such as TNF receptor ligation, p53 activation, oxidative stress, and the lipid second messenger sphingosine, Such a

translocation can also be induced by lysosomotropic agents such as cyprofloxacin, norfloxacin, and hydroxychloroquine. We also proposed lysosome as a proposed target for rose bengal in inducing cell death in melanoma cells (138).

We have previously found that rose bengal (a xanthine dye) could induces dual modes of cell death (apoptotic and non-apoptotic cell death) in melanoma cells and has clinical activity against melanoma (44). Recently, we showed apoptogenic properties of saffron (*Crocus sativus* L.), an Iranian medicinal plant, in human cancer cell lines and proposed saffron as a promising chemotherapeutic agent in cancer treatment (139).

Lysosomes and the endoplasmic reticulum (ER) hold promise as drug targets and mediators of apoptosis signaling which may be less affected by intrinsic or chemotherapy-induced resistance mechanisms. Tumor cell lysosomes contain increased levels of cathepsins, and the release of these enzymes into the cytosol may result in apoptosis or necrosis, as has been reported for TNF-α. It is also reported that tumor transformation leads to increased sensitivity to cathepsin B-dependent apoptosis (140)

Tumor cells often show evidence of constitutive ER stress, possibly due to hypoxia and glucose depletion. Various anticancer drugs, including cisplatin, tunicamycin and proteasome inhibitors, have been shown to induce ER stress. Manipulating the ER stress response of tumor cells is an interesting therapeutic strategy (73). We conclude that organelle damage responses can be used to trigger tumor cell death, and that the response to such damage may be triggered in cells that are resistant to conventional DNA-damaging agents (68)

Apoptosis and autoimmunity

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 (141). Autoimmunity represents a diverse set of diseases defined by the target organ destroyed. Apoptosis plays a prominent role in autoimmune diseases in two different ways. First, controlled regulation of apoptosis is a normal part of T cell selection and education. Interruption of this process could lead to auto-reactive cells and second, cell death can represent a lymphocyte-independent mechanism causing premature death of certain organs.

Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by the involvement of multiple organs and the presence of uto-antibodies against various nuclear and cytoplasmic antigens in serum (142). One common feature of SLE is the generation of antiphospholipid antibodies (143). Lymphocytes from lupus patients undergo accelerated apoptosis compared with normal individuals. Deficiencies in components of the complement cascade have been shown to predispose humans and mice to lupus-like diseases (144-147), as well as defects in certain pro- and anti-apoptotic molecules such as Fas and members of the Bcl-2 family (148;149), CD28 and CD40 (150-152). For example, the BH3-only protein Bim promotes apoptosis by binding to, and antagonizing Bcl-2 and Bcl-XL. In Bim−/− mice, plasma cells accumulate, inducing autoimmune kidney diseases (153). Moreover, DNase 1 deficient mice developed also lupus-like autoimmune disease (154).

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a systemic autoimmune disease whose hallmark is the estruction of the synovial membrane due to inflammatory and proliferative processes. At the cellular level, there is a remarkable hyperplasia of synoviocytes, in addition to local secretion of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α. Several reports suggest that defects in apoptosis regulation are implicated in the pathogenesis of RA. The RA synovium has an abnormal high proliferative rate, which may be explained by altered expression of anti-apoptotic genes (Bcl-2, Bcl-XL and survivin), oncogenes or tumor

suppressor genes (p53). In addition, the Fas/FasL pathway may also be implicated in RA, since both are constitutively expressed in RA (155-158). Various promising drugs such as Pralnacasan, HMR-3480, AZQs or SPC-839 that target either different types of caspases or the inhibitor of kappaB kinase (IKK) are currently tested in cellular models of RA and in clinical phase II trials (Table 5).

Autoimmune diabetes

Autoimmune diabetes, or insulin-dependent diabetes mellitus (IDDM), is characterized by selective destruction of insulin-producing cells. Infection-associated molecular mimicry has been a popular hypothesis for the development of autoimmunity in Type 1 diabetes. However, there is evidence that early developmental remodeling and/or homeostasis of β-cell mass involves β-cell apoptosis which might trigger autoimmunity. There is emerging evidence that T cell-induced apoptosis is a dominant effector mechanism in Type 1 diabetes.

Table 5. Novel promising therapeutics modulating apoptosis signaling pathways in inflammation, neurodegenerative diseases and cancer (11, 50).

Drug	Molecular target	Target disease
Pralnacasan	Caspase-1/-4 inhibitor	Rheumatoid arthritis (phase II)
AZQs (AstraZeneca)	Caspase-3 inhibitor	Rheumatoid arthritis and other inflammatory diseases
HGS-ETR1	Agonistic TRAIL-R1 mAb	Apoptosis induction in various tumor cell lines and tumor xenografts, synergistic with anticancer drugs (phase II)
HGS-ETR2	Agonistic TRAIL-R2 mAb	Apoptosis induction in tumor cell lines (phase 1)
HGS-TR2J	Agonistic TRAIL-R2 mAb	Apoptosis induction in tumor cell lines (phase 1)
PRO1762	Soluble human Apo2L/TRAIL	Apoptosis induction in tumor cell lines, no side effects in cynomolgus monkeys and mice, synergistic with anticancer drugs (phase 1)
AEG35156/GEM640	XIAP antisense oligonucleotide	Exhibits antitumor activity alone or in combination with chemotherapeutics in cancer xenograft models (phase 1)
LY2181308	Survivin antisense construct	Preclinical studies show antitumor activity in a broad range of cancers (phase 1 clinical trials started November 2004)
Cladribine	Direct disruption of mitochondrial membrane potential	Approved for chronic lymphocytic and hairy cell leukemia
Arsenite	Oxidative disruption of mitochondrial membrane and proteins	Approved for acute promyelocytic leukemia
IDN6556	Pan-caspase inhibitor	Prevents from cold- and ischemia-induced damage of donor liver organ transplants (phase II); multiple sclerosis; Hepatitis C (phase II)
SPC-839	IKK inhibitor	Arthritis
Minocycline	Inhibits cytochrome *c* release, NO-synthetase and casp-3 mRNA upregulation	ALS (phase III), HD (phase II), PD, multiple sclerosis
Pifithrin	p53 inhibitor	Nervous system trauma, stroke
Recombinant Trail	Activation of DR4 and DR5	Cancer (phase I)
Genasense	Bcl-2 antisense	Malignant melanoma (phase III), chronic lymphocytic leukemia (phase III), multiple myeloma (phase III)
Herceptin	Antibody blocking EGF-R (Her2/neu)	Metastatic breast cancer (approved)
INGN201	p53-expressing adenovirus	Apoptosis induction in tumor cell lines and xenograft models, head and neck cancer (phase III), clinical trials for other advanced solid tumors
SCH58500	p53-expressing adenovirus	Apoptosis induction in tumor cell lines and xenograft models, advanced ovarian cancer (phase III)
ONYX-015	p53 delivery with mutant adenovirus	Combination therapy of advanced squamous cell cancer (phase II and III);
IDN13389	XIAP antagonist	Cancer
Zarnestra	Farnesyltransferase inhibitor blocking Ras function	Acute myeloid leukemia (continuous marketing application in-process), multiple myeloma (phase II)

If clinical stage is not mentioned, drugs are at preclinical stage.

In this regard, pancreatic β-cells derived from newly diagnosed patients with Type 1 diabetes were found to have increased cell surface expression of Fas (CD95) as compared to β-cells from healthy subjects that did not constitutively express detectable Fas (CD95) (159). A murine model of IDDM, the nonobese diabetic mouse (NOD) spontaneously develops IDDM in two phases: infiltration of T and B cells, macrophages and dendritic cells and finally, destruction of β cells by CD8 and CD4 cells. Apoptosis of β cells has been clearly demonstrated in mouse models of IDDM. The proposed mechanism to explain the observed apoptosis included perforin-dependent cytotoxicity as well as TNF-α and Fas/FasL pathways (155, 156, 160-164). In addition, disruption of STAT4 activation completely prevents the development of spontaneous diabetes in NOD mice, suggesting an important role of STAT4 in autoimmune diabetes pathogenesis (165).

Multiple sclerosis

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) in which myelin and myelin-producing cells become the target of an inflammatory response leading to apoptotic cell death (166, 167). One of the most important progresses in the treatment of MS has been the development of interferon-β as a therapy. This therapy improves MS, possibly by lowering IFN-γ secretion and inhibiting responses to IFN-γ. IFN-β acts primarily on blood cells with probable selectivity for functionally different lymphocyte subpopulations, monocytes and granulocytes by downregulating FLIP expression (168). In addition, upregulation of Bcl-2, CD95 and CD95L has been observed in MS patients. Abnormal Bcl-2 expression may promote apoptotic resistance of potentially pathogenic autoreactive lymphocytes and may allow for continuing cellular proliferation and tissue destruction. (169, 170). It has demonstrated that members of the inhibitor of apoptosis (IAP) family of anti-apoptotic genes are elevated in peripheral blood immune cells (monocytes, T cells) of patients with aggressive forms of MS These findings suggest that the IAPs may be novel diagnostic markers for distinguishing subtypes of MS. Moreover, antisense-mediated knockdown of the IAP family member known as X-linked IAP (XIAP) reverses paralysis in an animal model of MS suggesting that treatments targeting XIAP may be useful in the treatment of MS (171).

Apoptosis and neurodegenerative disorders

Neurodegenerative diseases include a variety of progressive disorders resulting in cognitive and/or motor deterioration. For such disease, it is clear that apoptosis mechanisms are the candidates for cell death. Excessive death of one or more populations of neurons results in disease or injury. For example, death of hippocampal and cortical neurons results in Alzheimer's disease (AD), death of mid brain neurons results in Parkinson's disease (PD), death of neurons in the stratium results in Huntington's disease (HD) and finally, death of lower motor neurons results in amyotrophic lateral sclerosis (ALS). We have reported that glucose could induce apoptosis in PC12 cells as a possible mechanism of glucose-induced neuropathy in diabetes (104). In an other study using PC12 cells, we have shown lead could cause PC12 cell death, in which apoptosis or programmed cell death plays an important role (105).

Parkinson's disease

PD patients suffer from degeneration of dopaminedependent neurons in their substantia nigra. Increase oxidative stress and mitochondria dysfunction seem to be the central key of this disease (172). Evidence for apoptosis in PD has been observed in human tissues, as well as in animal models (173-175). Dopaminergic neurons die by apoptosis as shown by histochemical evidences (176) and increased expression of apoptosis-related genes encoding p53,CD95 and Bax, as well as Par-4, has been observed in brain tissue from PD patients (177, 178). Furthermore, it has been shown that delivery of an Apaf-1 dominant negative mutant using an adenovirus vector in a mouse model of PD inhibits mitochondrial apoptotic signaling pathways

preventing neuronal cell death. This report suggests that gene therapy may be an encouraging approach for treatment of neurodegenerative disorders (173). In addition, Cephalon Inc. and Lundbeck have discovered a novel drug named CEP-1347 offering great benefits for PD patients in phase II trials by blocking mixed-lineage kinases of the JNK pathway.

Alzheimer's disease

Alzheimer's disease (AD) correlates with synaptic degeneration and death of neurons in limbic structures (179, 180). A defining feature of AD is the accumulation of amyloid plaques formed by aggregates of the amyloid-β peptide (80, 181, 182), a fragment generated by processing of amyloid precursor protein (APP). Increased DNA damage, caspase activity and altered expression of Bcl-2 family members have been demonstrated in neurons associated with amyloid deposits (183, 184). Moreover, caspase-mediated cleavage of APP results in the release a carboxy-terminal peptide, which is a potent inducer of apoptosis (185-187). Recent advances in the molecular genetics of AD have led to the identification of four specific genes involved in the disease: β-amyloid precursor protein (β-APP), presenilin-1 (PS1), presenilin-2 (PS2) and apolipoprotein E (185, 186). It is now clear that mutations, or unfavorable forms of these genes results in increased P-amyloid plaques in the brain. Studies of human tissues of AD prove that expression of the anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax are regulated in areas of the brain showing increased apoptosis (188). Addition of β- amyloid peptide to PC12 cells, neuroblastoma cells, rodent cortical and hippocampal neurons results in apoptotic cell death (189-191). Altered proteolytic processing of APP is shown to be an important alteration contributing to the neurodegenerative cascade. Oxidative stress and perturbed regulation of intracellular calcium levels are central to the neuronal death in AD. Additional biochemical data have proposed that mitochondrial function is compromised in brain cells of AD patients (192). Analyses of post-mortem brain tissues from AD patients have provided evidence for nuclear DNA fragmentation. Immunohistochemical studies revealed elevated levels of caspase activity (193), increased expression of apoptosis-related gene Bax (194), as well as elevated levels of Par-4. Similarly, Huntington's disease (HD) is associated with mitochondrial dysfunction and increased caspase-2 activation (195).

Prospects of Apoptosis-Targeted Therapies

Depending on the molecular target, different strategies are being employed. Recombinant biologicals including death ligands or agonistic and antagonistic antibodies that inhibit or trigger death receptor signaling have proven efficacy in various animal models. Although, new drugs are currently being designed the relatively low rate of clinical entry associated with these molecules is related to the lack of specificity, low efficacy, or development to drug resistance. Application of various mechanisms at which apoptosis can be targeted offers hope that apoptosis-based therapies and improved clinical outcome for a wide range of diseases may be not far from realization (196).

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