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Signaling Pathways and Effector Mechanisms Pre-Programmed Cell Death

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Abstract—Apoptosis is a complex biochemical process that involves all aspects of the cell from the plasma membrane to the nucleus. Apoptosis stimuli are mediated by many different cellular processes including protein synthesis and degradation, the alteration in protein phosphorylation states, the activation of lipid second messenger systems, and disruption of normal mitochondrial function. Despite this diversity in signal transduction, all apoptotic pathways are believed to converge ultimately with the activation of caspases leading to the characteristic morphological changes of apoptosis. In this review, we discuss what is known about these pathways and its implication for normal cellular function.

Introduction

The term apoptosis, which first appeared in 1972, was used to describe a new pattern of cell death.[1] In apoptosis, cells undergo characteristic morphological changes including condensation and fragmentation of the nucleus, shrinkage of the cytoplasm, and the formation of what are termed apoptotic bodies that contain self-enclosed fragments of the nucleus surrounded by cytoplasm and a cell membrane (Fig. 1).[1,2] Apoptotic cells are rapidly engulfed by phagocytic cells including macrophages.[3] In part because their plasma membrane remains intact, apoptotic cells do not trigger an inflammatory response.[4] These features of apoptosis stand in contrast to the features of necrosis, the prevailing form of cell death resulting from a non-specific injury such as blunt trauma, exposure to a toxin, or the loss of blood supply.[1,2,5] In necrosis, cells often undergo swelling and eventual rupture. The release of cytoplasmic contents, in addition to other events, triggers a pronounced inflammatory response.[2,4]

After the initial description of apoptosis, it was recognized that apoptosis occurs in all tissues as part of normal cellular turnover.[1] Apoptosis also occurs during embryogenesis in which particular cells are 'programmed' to die, and hence the term 'programmed cell death' is used to describe this process.[6,7] Initially, programmed cell death referred to the biochemical process of apoptosis, and apoptosis referred to the morphological changes. However, apoptosis has since come to be used to describe both processes.[8]

Apoptosis can be divided into three stages.[9] In the first stage, the cell receives an apoptotic signal. A variety of stimuli both internal and external to the cell can activate apoptotic pathways. These include ligation of a cell surface receptor, removal of essential growth factors, or exposure to various chemical agents.[10] In addition, the exposure of a cell to UV or ionizing radiation, heat, and changes to osmolarity can all induce apoptosis.[11–15] In the next stage of apoptosis, the cell integrates the various signals and may, or may not commit to apoptosis. This process involves several signal transduction pathways such as the activation (or inactivation) or serine/threonine and tyrosine kinases and phosphatases, the synthesis of lipid second messengers including ceramides, altered gene expression, and the activation of specialized proteases known as caspases (see below). The final decision to undergo apoptosis depends upon several factors including the relative levels of both apoptotic and survival factors (including members of the Bcl-2 family of proteins), as well as the metabolic state of the cell, and can be influenced by what stage of the cell cycle the cell is in.[16–18] Recent experiments have indicated that many apoptotic stimuli are integrated by mitochondria.[9] In the final stage of apoptosis, a common degradative/signaling pathway is activated that triggers the acquisition of the characteristic morphological features.[9]

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Features of apoptosis

The biochemical and morphological changes caused by apoptosis affect all aspects of the cell from the plasma membrane to the nucleus (Table 1). The plasma membrane is composed of a variety of phospholipids including both aminophospholipids (phosphatidylserine, PS, and phosphatidylethanolamine) and choline phospholipids (phosphatidylcholine, sphingomyelin).[19] In the normal cellular state, the cell maintains an asymmetry between the phospholipid content of the inner and outer leaflet of the plasma membrane by actively translocating PS from the outer to the inner leaflet. In apoptosis, this asymmetry is lost as PS equilibrates between the inner and outer leaflets, a process that is facilitated by a Ca^{2+}-dependent flip-flop.[20,21] The presence of PS on the outer cell surface can be detected using the PS-binding protein, annexin-V, and is a sign for neighboring cells and macrophages to remove the apoptotic cell.[3,19] Another prominent apoptotic change affecting the structure of the plasma membrane is the formation of blebs, or small, membrane-enclosed pieces of cytoplasm and condensed nuclear material.[5] One of the final signs to appear during apoptosis is the inability of the plasma membrane to exclude dyes (e.g., trypan blue, ethidium bromide, and propidium iodide) which provides a convient way to monitor cell death.[22–24]

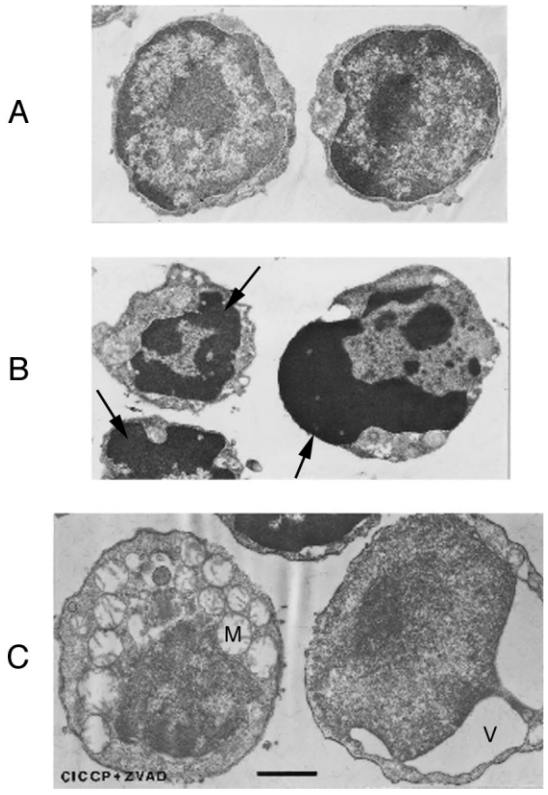

Figure 1. Morphology of apoptosis and necrosis. Electron microscopic morphology of BALB/c thymocytes. Apoptosis (panel B) was induced by culture (6 h) with the mitochondrial-damaging agent carbonyl cyanide *m*-chlorophenyl hydrazone (mClCCP) (100 μM). Note the prominent chromatin condensation present in these cells (indicated by the arrows). Apoptosis was inhibited, but necrosis occurred (Panel C) when the thymocytes were incubated with mClCCP and the caspase-inhibitor z-VAD.fmk (50 μM). These cells do not show any chromatin condensation but do have other features of necrosis including disruption of the plasma membrane, the formation of vacuoles (V), and pronounced mitochondrial swelling (M). The bar in the necrosis image equals 1 μm. This figure has been adapted from Hirsch et al.[33]

Cytoplasmic changes during apoptosis include the loss of cytoplasmic volume and degradation of cytoskeletal proteins.[5] Cytoplasmic shrinkage results primarily from two processes. The formation of blebs results in a loss of volume. In addition, the loss of a potassium gradient that is normally maintained by the plasma membrane results in osmotic shrinkage of the cytoplasm.[25] The degradation of cytoskeletal proteins results from the actions of caspases both directly cleaving proteins and activating other degradative enzymes.[7]

In its normal state, the mitochondrion is an oblong structure with both an inner and outer phospholipid membrane (Fig. 2).[26] The inner membrane defines the central matrix space where mitochondrial DNA is housed and where protein synthesis, respiration, and the biosynthesis of steroid hormones occurs.[26] The inner and outer membranes also define an inter-membrane space that contains several pro-apoptotic proteins including the respiratory protein cytochrome c, apoptosis-inducing-factor (AIF), and in some cells, caspases 2, 3, and 9 have also been detected.[27–32] Mitochondria normally maintain a voltage ($\Delta\Psi_m$) and pH gradient across the inner membrane that is used by the F_1F_0-ATPase in the formation of ATP.[26] During apoptosis,

Table 1. Features of apoptosis

Structure	Alteration	Measurement
Plasma membrane	Exposure of phosphatidylserine	Annexin-V binding
	Membrane blebbing	Microscopy
	Loss of integrity	Inclusion of propidium iodide, trypan blue
	Loss of potassium gradient	
Cytoplasm	Loss of cytoplasmic volume	Microscopy
	Degradation of cytoskeletal proteins	
Mitochondria	Rupture of outer membrane	Microscopy
	Swelling of matrix	Microscopy
	Release of apoptotic proteins	Immunodetection
	Loss of transmembrane gradient	Lipophilic cationic dyes ($DIOC_6(3)$, JC-1, R123)
Nucleus	Nuclear fragmentation	Microscopy
	Chromatin condensation	Microscopy
	DNA fragmentation	TUNEL, electrophoresis, total DNA content

this gradient is often lost, and is associated with three other mitochondrial alterations that occur during apoptosis: osmotic swelling of the matrix, rupture of the outer mitochondrial membrane, and the release of pro-apoptotic proteins from the inter-membrane space.[9]

Mitochondrial swelling is not limited to apoptotic processes. As can be seen in Figure 1, pronounced mitochondrial swelling can occur with necrotic processes.[33] In contrast to necrosis, mitochondrial swelling during apoptosis is usually only a transient phenomenon. During apoptosis, shrinkage of cytoplasmic volume secondary to the loss of the plasma membrane potassium gradient results in increased cytoplasmic osmotic pressure. This increased osmotic pressure is believed to prevent sustained mitochondrial swelling.[25]

In its normal state, the nucleus is a spherical structure with a relatively diffuse staining pattern. This staining pattern reflects different chromatin states with darker regions indicating inactive stretches of DNA bundled together with histones.[34] Using microscopy, several dramatic alterations are observed as a result of apoptosis. These changes include condensation of the nucleus such that it occupies less space and stains more intensely.[2] In addition, the nucleus often fragments into several sections.[5] These sections can also be released from the cell as part of apoptotic bodies.[35] Hence, a reduction in the total DNA content of the cell is a sign of an apoptotic cell death. On a biochemical level, these nuclear changes are associated with the activation of several different endonucleases. The action of the DNA degradative enzymes results in the formation of small fragments of DNA, often in multiples of 180 base-pairs reflecting basic nucleosome structure.[24,35]

Caspases

Caspases are specialized proteases that are essential for apoptosis. They are distinct from other proteases because they use a cysteine for catalysis and only cleave after aspartic acid residues.[36] This unusual aspartate substrate specificity is only found in one other protease, granzyme B, though this enzyme uses an active site serine.[12,37] Caspases are synthesized as a single polypeptide chain, and are inactive zymogens. During activation, each polypeptide chain is cleaved into a large and small subunit, which then dimerize.[7] Two large and two small subunits are required for full enzymatic activity.[7] Activation occurs by one of two mechanisms. Some caspases are triggered to undergo self-cleavage by interaction with other proteins. For example, caspase-8 is activated as a result of its interaction with Fas-associated death domain (FADD),[38,39] and caspase-9 is activated through an interaction with cytochrome c, dATP (or ATP), and apoptotic protease activating factor-1 (Apaf-1).[40] In the other mechanism, caspases are activated by other caspases. For example, both active caspase-8 and caspase-9 can cleave and activate caspase-3.[40]

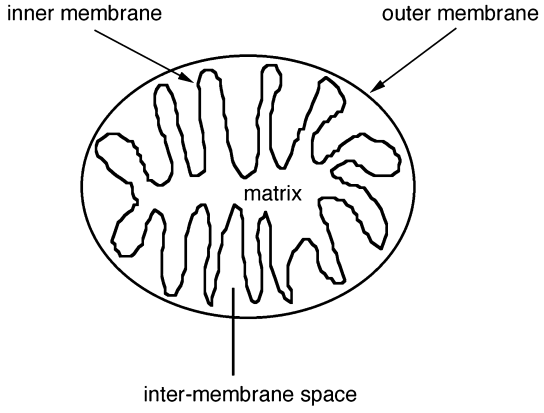

Figure 2. Structure of the mitochondrion.

At least 14 different caspases have been identified in mammalian tissues.[41] Based on substrate specificity for the three amino acids preceding the aspartic acid, caspases can be segregated into three different categories (Table 2). The first group (group I) contains caspases involved in inflammatory processes including caspases 1, 4, and 5.[42] These enzymes are sometimes known as ICE-like caspases, because another name for caspase-1 is Interleukin-1-converting enzyme (ICE). Their preferred tetrapeptide recognition motif is WEHD, though the ICE-like caspases are more tolerant of amino acid substitutions than the signaling or effector caspases.[43] The second category (group II) of caspases contains caspases 6, 8, 9, and 10.[43] These enzymes are considered signaling caspases because they can activate other caspases, initiating a cascade.[43] The final category (group III) contains caspases 2, 3, and 7. These enzymes are known as effector caspases because they are activated by other caspases, and because they cleave many cellular targets resulting in the acquisition of apoptotic morphology. Activation of these caspases generally results in an irreversible commitment to cell death.[41,44] The effector enzymes are the most specific, with a near absolute requirement for an aspartic acid in the first and fourth position prior to the cleavage site.[43] The most recently identified caspases, 12–14, have not yet been sufficiently characterized to place them in one of these three categories.

Caspases are responsible for cleaving numerous cellular targets, including structural elements, nuclear proteins, and signaling proteins (Table 3).[7] Cytoskeletal proteins cleaved by caspases include lamin, α-fodrin, and actin.[7] It is possible that cleavage of these proteins is associated with the dramatic morphological alterations seen in apoptosis.[7] Caspases have been shown to cleave and inactivate many nuclear elements including the U1 (70 kDa) ribonuclear protein involved in RNA splicing and two enzymes involved in DNA repair, poly(ADP-ribose) polymerase (PARP) and DNA-dependent

Table 2. Caspase categories

Category	Caspases	Recognition motif
ICE-like	1, 4, 5, 11	WEHD
signaling	6, 8, 9, 10	(LV)EXD
effector	2, 3, 7	DEXD
	12, 13, 14	Unknown

X indicates that any amino acid is tolerated.

Table 3. Caspase Targets

Category	Target	Outcome
Signaling	Other caspases	Activation
	PKC δ	Activation, nuclear fragmentation
	p21-Activated kinase	Activation of JNK
	PISTRLE kinase	Activation
	Phospholipase A_2	Activation
	Bcl-2, Bcl-x_L	Inactivation
	Bid	Activation
Nuclear	DNA fragmentation factor	DNA fragmentation
	Inhibitor of caspase-activated DNAse (CAD)	Activation of CAD, DNA fragmentation
	Poly (ADP-ribose) polymerase	Inactivation
	DNA-dependent protein kinase	Inactivation
	U1 (70 kDa)-snRNP	Inactivation
Structural	Lamin	Degradation
	α-Fodrin	Degradation
	Actin	Degradation

protein kinase (DNA-PK).[7,44–47] Caspases also activate two factors that lead to DNA fragmentation. These include a DNA fragmentation factor and an inhibitor of the caspase-activated DNAse.[7] Many caspase targets are themselves involved in signaling processes. Proteins falling into this category include other caspases, several kinases, and phospholipase A_2.[7] In addition, caspases can cleave members of the Bcl-2 family of proteins.[48]

Caspase activation and enzymatic activity can be inhibited by several mechanisms. The most common technique is to pretreat cells/enzymes with a chemically modified tri- or tetrapeptide containing an aspartate as the C-terminal amino acid. Using strategies developed for inhibition of other proteases, aldehyde-modified (-CHO) peptides are reversible caspase inhibitors, while fluoromethyl ketone-modified peptides are irreversible inhibitors.[43] By using different peptide sequences, it is possible to target different groups of caspases. For example, the tripeptide benzyloxycarbonyl-VAD-fluoromethyl ketone (z-VAD-fmk) effectively inhibits all caspases (although it prefers caspase 8), and the tetrapeptide *N*-acetyl-DEVD-CHO inhibits group II and III caspases, but is less active against group I caspases.[43] In contrast, *N*-acetyl -WEHD-CHO and *N*-acetyl -YVAD-CHO inhibit group I caspases better than group II, and are ineffective against group III caspases.[43] Group I and II caspases can also be inhibited by transfecting cells with the DNA sequence for the cowpox serpin CrmA.[43,49] Distinct from these peptide-based strategies, caspase activity is also modified by phosphorylation. Phosphorylated caspase-9 is resistant to activation, and once activated the phosphorylated enzyme has decreased activity.[50]

Bcl-2 family of proteins

Bcl-2 and its related proteins are important modulators of apoptosis. This family of proteins includes members that inhibit apoptosis (Bcl-2, Bcl-x_L, Mcl-1, A1) and promote apoptosis (Bax, Bak, Bad, Bid, Bik, Bcl-x_S).[51,52] Based on sequence alignment, these proteins contain up to four Bcl-2-homology (BH) domains.[51] All of the anti-apoptotic members contain all four domains, while the pro-apoptotic members can be divided into three categories.[53] One group contains BH1, BH2, and BH3 (Bax, Bak), while another group only contains the BH3 domain (Bad, Bid, Bik).[51] Bcl-x_S forms its own category, containing both BH3 and BH4 domains.[52] The structure of the Bcl-x_L protein has been solved by NMR and crystallography.[52] This protein consists of two central α-helices surrounded by five amphipathic α-helices. The sequences for the α-helical regions are located in the BH domains. Interestingly, this three-dimensional structure is homologous to membrane pore-forming bacterial toxins, suggesting a potential mechanism of action for these proteins.[52] Another structural characteristic of Bcl-2 proteins is the capability for both homo- and heterodimerization. In particular, it has been observed that heterodimerization between pro- and anti-apoptotic family members involves the BH3 domain α-helix, and can modulate their activity.[52]

Different Bcl-2-like proteins have different cytoplasmic distributions. Both Bcl-2 and Bcl-x_L have a C-terminal membrane insertion sequence, and the majority of these proteins are found to be associated with the membranes of the mitochondria, endoplasmic reticulum, and nucleus.[52,54,55] In their inactive state, the pro-apoptotic members, Bad, Bax, and Bid, have primarily a cytoplasmic location. However, upon activation, they translocate to the mitochondria.[54,56,57] The action of Bcl-2-like proteins in both preventing and initiating apoptosis is currently believed to reside with the mitochondria, although the precise mechanism of action remains unclear and controversial. Several family members can interact with the adenine nucleotide translocator (ANT) and voltage-dependent anion channel (VDAC).[58,59] As will be discussed below, these two proteins are part of the mitochondrial permeability transition (PT) pore that is believed to control both the release of pro-apoptotic cytochrome c and the mitochondrial transmembrane voltage gradient. In cell-free systems, Bcl-2 and Bcl-x_L favor closure of PT pore, while pro-apoptotic Bax has the opposite effect, interacting with ANT and VDAC to favor pore opening and cytochrome c release.[58–61] The mechanism by which BH3-only proteins induce mitochondrial apoptosis is not as straightforward. Bid and Bik can directly induce mitochondria to release

cytochrome c, however, they do not interact with either ANT or VDAC, suggesting that they are acting outside of the PT pore.[58] Bid, Bik, and Bad may also act to inhibit the anti-apoptotic actions of Bcl-2 and Bcl-x_L via the formation of heterodimers.[58]

Several different cellular mechanisms exist to modulate the activity of both the pro- and anti-apoptotic function of Bcl-2 proteins. First, the dimerization state of Bcl-2 family members affects their activity.[51] One function of the anti-apoptotic Bcl-2 and Bcl-x_L is to dimerize with pro-apoptotic Bax to neutralize its activity. As a heterodimer, Bax is inactive, but once free to dimerize with itself, Bax is able to induce apoptosis.[52,62] Second, altering the expression levels of pro- and anti-apoptotic family members can either promote or inhibit apoptosis.[63] For example, when the amount of Bcl-2 is greater than or equal to the amount Bax, a given cell is protected from apoptosis. However, when the amount of Bax exceeds the amount Bcl-2, a cell is more prone to undergo apoptosis. Third, Bcl-2 proteins can be altered by phosphorylation.[53,64,65] The best example of this concerns the pro-apoptotic protein Bad. In its unphosphorylated state, it dimerizes with Bcl-2 and Bcl-x_L, neutralizing their anti-apoptotic activity (and allowing Bax to self-associate).[66] However, when Bad is phosphorylated, it is sequestered by a 14-3-3 protein, and therefore cannot interact with and neutralize Bcl-2 and Bcl-x_L.[51,67] Fourth, the function of Bcl-2 family members can be altered by cleavage. During Fas-mediated apoptosis, caspases have been shown to cleave both Bcl-2 and Bcl-x_L.[48,68] The cleaved products are no longer protective, and in fact become pro-apoptotic.[48,68] Bid is another Bcl-2 protein that is activated by caspase cleavage. While the full-length protein is inactive, after caspase-8-mediated cleavage, Bid induces cytochrome c release from the mitochondria.[69–71] Finally, the conformation of Bcl-2 proteins modifies their activity. The best evidence for this mechanism comes from studies of Bax.[57] In its inactive state, Bax exists in a conformation in which it is resistant to protease cleavage. However, upon activation and translocation to mitochondria, the N-terminal region of this protein becomes susceptible to protease cleavage, suggesting that a conformational change has occurred.[57] In vitro studies have indicated that Bax undergoes a conformational change that is controlled by pH.[57] At neutral pH, Bax is resistant to cleavage, however, at basic pH, Bax become sensitive. Furthermore, in whole cell systems, Bax activation during apoptosis is preceded by a transient rise in intracellular pH.[57]

Apoptotic death pathways

Diverse stimuli can initiate apoptosis, however common biochemical and morphological alterations are observed independent of the initial stimulus. This finding suggests that most apoptotic signals converge on a limited number of common effector pathways.[72] On a basic level these pathways can be distinguished by the relative timing of caspase activation and the mitochondrial release of cytochrome c. In one pathway, which is exemplified by the activation of death receptors, an effector caspase is activated prior to mitochondrial alterations.[7] Oligermization of these receptors leads to activation of caspase-8 which then initiates a caspase cascade leading to the activation of caspase-3 and cell death (Fig. 3). In the other pathway, cytochrome c is released from the mitochondrial intermembrane space prior to caspase activation.[9] As mentioned above, the complex of cytochrome c, Apaf-1, dATP, and pro-caspase-9 activates caspase-9. Caspase-9 then activates caspase-3, triggering a commitment to apoptosis (Fig. 3). While these two pathways are presented as separate, several mechanisms exist for cross-talk and positive feedback loops. For example, caspases can cleave Bcl-2, Bcl-x_L, and Bid leading to mitochondrial apoptosis.[48,68–71] In addition, activated caspase-3 can activate caspase-6 which in turn activates caspase-8.[73]

Death receptors

Death receptors are cell surface receptors so named because their ligation can trigger apoptosis. These receptors, which are members of the nerve growth factor receptor family, include Fas (CD95/APO-1), tumor necrosis factor receptor 1 (TNF-R1), as well as DR-3, DR-4, and DR-5.[7,74] They are activated by both cell surface bound and soluble ligands such as FasL (CD95L), tumor necrosis factor-α (TNF-α), lymphotoxin-a (LT-α),

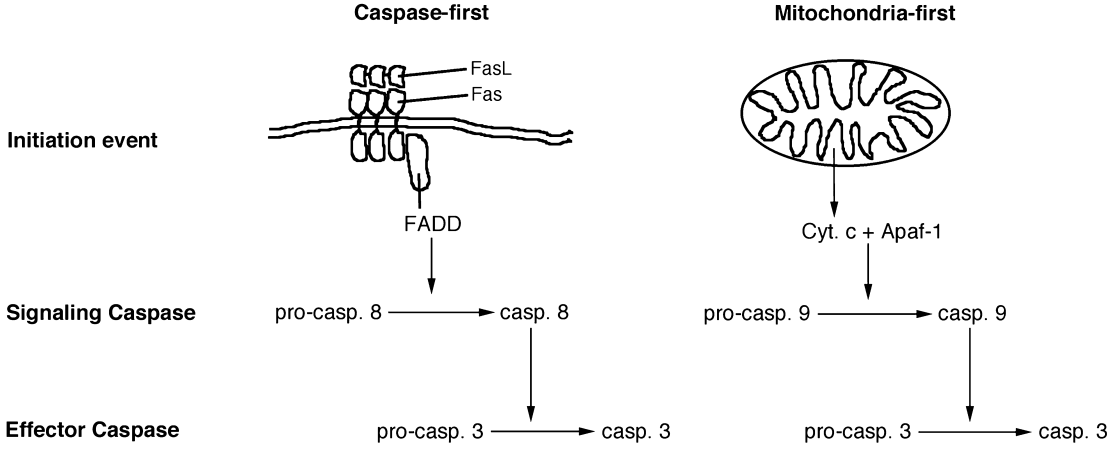

Figure 3. Apoptosis pathways.

and TNF-related apoptosis inducing ligand (TRAIL).[7,74] Death receptor ligands are members of the TNF-α cytokine family, and are homotrimeric molecules.[7] Crystallographic analysis of the LT-α bound to TNF-R1 reveals that each monomer binds to one receptor, indicating that surface ligand binding leads to trimerization of these receptors.[74]

Activation of these receptors leads to apoptosis via several protein–protein interactions. The cytoplasmic parts of these receptors contains a region known as the death domain (DD).[7,74] As a result of surface ligation, the intracellular DDs trimerize, thereby recruiting and dimerizing with adaptor molecules. These adaptor molecules include FADD, TNF-R1-associated protein with death domain (TRADD), and the receptor interacting protein death domain RIP-DD.[7,74] As indicated by their name FADD, TRADD, and RIP-DD contain their own DDs. Focusing on Fas as a model system, the complex of Fas and FADD recruits pro-caspase-8 forming the death-inducing signal complex (DISC).[7,74] Once assembled, the DISC triggers the rapid self-activation of caspase-8, and initiates a caspase-first apoptosis pathway (see Fig. 3).

In addition to the direct activation of caspase-3, activation of the Fas receptor leads to several additional pro-apoptotic signaling events. First, caspase-8 triggers the formation of ceramide (see below).[75] While there is some debate about the precise sequence of events leading to ceramide formation, one potential pathway involves activation of a phosphorylcholine-specific phospholipase C.[76] This enzyme forms diacylglycerol (DAG) that then activates a sphingomyelinase that produces ceramide.[77] Second, caspase-8 can cleave Bid, a pro-apoptotic Bcl-2 family member.[69–71] Activation of Bid causes the release of cytochrome c from the mitochondria and activation of the mitochondria-dependent apoptosis pathway.[69–71] Third, Fas activation leads to the activation of c-jun NH_2-terminal kinases (JNK) and p38 mitogen activated protein kinases (MAPK).[78–80] While the precise steps leading to the activation of these enzymes is not clear, it may include the p21-activated kinase (PAK2), ceramide, and/or the Ras-family of kinases.[7,75,78]

The relative importance of each signal generated by ligation of Fas depends upon the cell type and initial apoptotic stimulus. For example, in some cell types, Fas ligation leads almost exclusively to a caspase only pathway (type I cells). These cells do not generally show mitochondrial involvement, and cell death is not inhibited by Bcl-2 or Bcl-x_L. Most cell types that have been examined belong to this category including the B-cell lines SKW6.4, Raji, BJAB, CH1, BL60 (K50), and HT29; the T-cell lines H9 and HuT78; the HepG2 hepatoma cell line; the human colon carcinoma cell line HT29; and the murine fibroblast cell line L929.[72,81] In addition, both thymocytes and peripheral T cells are believed to be type I cells.[81] In other cells, Fas ligation triggers an almost exclusive mitochondrial pathway after the activation of caspase-8. Hence, in these cells (type II), Bcl-2 and Bcl-x_L inhibit cell death via their actions at preventing the release of mitochondrial cytochrome c. Examples of type II cells include the Jurkat and CEM T cell lines, the P815 mouse mastocytoma cell line, and primary liver cells.[72,81] Recent experiments have suggested that the distinction between type I and type II cells may be an artifact resulting from the use of a non-physiological ligand. For example, when type II cells are incubated with an anti-Fas monoclonal antibody, a mitochondrial pathway results. However, when these same cells are incubated with FasL, little if any mitochondrial involvement is detected, and Bcl-2 and Bcl-x_L cannot inhibit cell death.[72]

Mitochondrial apoptosis

In death receptor-mediated apoptosis, the apoptotic pathway is activated by the interaction of a relatively small number of structurally-related ligands with a relatively small number of structurally-related cell surface receptors. In contrast, mitochondrial apoptosis can be induced by a variety of agents including chemotherapeutic drugs, reactive oxygen species, kinase and phosphatase inhibitors, respiratory poisons, Ca^{2+}-ionophores, UV- and γ-irradiation, granzyme B, and environmental stresses including growth factor withdrawal, heat, and osmolarity changes.[9,33,82–89] The varied nature of these apoptotic triggers suggests that it must be possible to induce mitochondrial apoptosis by more than one mechanism.

In an effort to understand how such diverse stimuli can induce mitochondrial-dependent apoptosis, the behavior of mitochondria in cell free systems has been studied extensively. Isolated mitochondria in these systems undergo what is referred to as the mitochondrial permeability transition (MPT).[90] Experimentally, the MPT is characterized by the abrupt increase in the permeability of the inner mitochondrial membrane to solutes with a molecular weight $\leq$1500 Da. This permeability transition has several consequences including the collapse of $\Delta\Psi_m$, osmotic swelling, matrix Ca^{2+} release, the generation of reactive oxygen species, and rupture of the outer mitochondrial membrane leading to the release of cytochrome c from the inter-membrane space. Biochemical characterization of mitochondria has identified a voltage- and Ca^{2+}-sensitive pore that controls the MPT, and is referred to as the PT pore.[9,90] This pore localizes to the junction of the inner and outer mitochondrial membranes, and therefore, opening of the pore allows for direct communication between the mitochondria matrix and the surrounding environment. The PT pore consists primarily of the ANT, the VDAC, and the PBR.[90] In addition, other molecules including cyclophilin D and Bcl-2 family members can be found in close association.[59] Opening of the PT pore is affected by several different factors including $\Delta\Psi_m$, oxidation state, matrix pH, matrix Ca^{2+}, and the conformation of the ANT (Table 4).[9,90,91] These factors interact to determine the probability of PT pore opening.

Examination of the relationship between the characteristics of mitochondrial-dependent apoptosis in whole cells and the MPT in isolated mitochondria finds

Table 4. Modulators of the mitochondrial permeability transition pore

Category	Function	Inducer	Inhibitor
$\Delta\Psi_m$	Loss of $\Delta\Psi_m$ favors pore opening	Respiratory uncouplers	
Oxidation state	Oxidation of pore thiols into a Disulfide causes pore opening	ROS	Free radical scavengers (e.g., butylhydroxytoluene, *N*-acetylcysteine)
Matrix pH	Pore opening is inhibited by acidic pH in the matrix		
Matrix Ca^{2+}	Increase in matrix Ca^{2+} favors pore opening	Ca^{2+}	RR
ANT conformation	Alteration of ANT conformation can either inhibit or enhance pore opening	Atractyloside	Bongkrekic acid
Bcl-2 proteins	Modulate PT pore opening	Bax	Bcl-2, Bcl-x_L
Cyclophilin D	Modulate PT pore opening		Cyclosporin A

$\Delta\Psi_m$, mitochondrial transmembrane potential; ROS, reactive oxygen species; RR, ruthenium red; an inhibitor of the uniporter responsible for matrix Ca^{2+} uptake; ANT, adenine nucleotide translocator.

striking parallels. Bcl-2 and Bcl-x_L inhibit apoptosis induced by mitochondria, and these two proteins also inhibit the MPT in isolated mitochondria.[9] Similarly, Bax induces both mitochondrial-dependent apoptosis and the MPT.[92–94] In whole cell systems, the production of ROS and the collapse of $\Delta\Psi_m$ is both a cause and consequence of apoptosis.[23,33,84] Likewise, the production of ROS and the collapse of $\Delta\Psi_m$ is both a cause and consequence of the MPT.[90,95,96] In addition, in cell free systems, supernatants from isolated mitochondria that have undergone the MPT can induce caspase activation and DNA fragmentation, while supernatants from intact mitochondria fail to cause any such apoptotic changes.[97] These observations have led to the hypothesis that mitochondrial apoptosis is caused by the MPT via the actions of the PT pore. Because of the ability of the PT pore to integrate many different cellular signals, this hypothesis provides a framework to understand how mitochondrial dependent apoptosis can be caused by so many different agents. In particular, some apoptotic stimuli directly modulate PT pore activity, while others are believed to act through second messengers. For example, ROS, Ca^{2+}-ionophores, and respiratory poisons that cause dissipation of $\Delta\Psi_m$ likely act through a direct effect on the PT pore.[90] In contrast, most chemotherapeutics, kinase/phosphatase inhibitors, granzyme B, UV- and γ-irradiation, and environmental stresses act via intermediaries that then directly act on the mitochondria.[9,85,98,99]

Because of the importance of cytochrome c in initiating the caspase cascade in mitochondrial apoptosis, researchers have examined the timing of cytochrome c release relative to other consequences of the MPT. The MPT hypothesis predicts that cytochrome c is released as a consequence of the MPT, and therefore cytochrome c release should either coincide with or follow other features of MPT including collapse of $\Delta\Psi_m$ and mitochondrial swelling. When Ca^{2+}, the ANT ligand atractyloside, or *tert*-butyl hydroperoxide are incubated with isolated mitochondria, these agents cause opening of the PT pore, and a nearly immediate collapse of $\Delta\Psi_m$.[96] Mitochondrial swelling and release of cytochrome c then occurs several minutes later.[96] Indeed, with Ca^{2+}-induced MPT, if the Ca^{2+}-chelator EGTA is added up to two minutes after the addition of Ca^{2+}, the $\Delta\Psi_m$ recharges, and both mitochondrial swelling and cytochrome c release do not occur.[96] These findings suggest that cytochrome c release occurs secondary to rupture of the outer membrane. However, this mechanism cannot explain the behavior of other inducers of the MPT. Experiments with isolated mitochondria using Bax and Bid have found that release of cytochrome c occurs independently of collapse of $\Delta\Psi_m$ and mitochondrial swelling.[58,93] In addition, in whole cell and cell-free systems, cytochrome c release often occurs prior to collapse of $\Delta\Psi_m$.[28,83,86,100,101] These findings suggest that cytochrome c release may not be a result of the MPT. In an effort to reconcile these discrepancies, Pastorino et al. have proposed that the inability to detect collapse of $\Delta\Psi_m$ and mitochondrial swelling prior to cytochrome c release is because detection techniques measure the bulk properties of population of mitochondria, and are not sensitive enough to measure the behavior of individual mitochondria.[94] Therefore, they propose that it is possible for agents to cause either transient opening of the PT pore sufficient to allow cytochrome c release but not $\Delta\Psi_m$ collapse, or that the PT pore is opened in only a fraction of the total mitochondrial population. While further experiments are needed to clarify the exact relationship between cytochrome c release, mitochondrial swelling, and collapse of $\Delta\Psi_m$, the ability of agents to induce apoptosis via opening of the PT pore and induction of the MPT remains a useful working model.

p53

The tumor suppressor p53 is a transcription factor that has a critical role in preventing cancer. Depending upon the stimulus and the state of the cell, activation of p53 can lead to either the halting of cellular proliferation and DNA repair, or to apoptosis.[102] While the primary stimulus for activating p53 is DNA damage,[103] other cellular stresses including metabolite deprivation, physical damage, heat shock, and loss of oxygen can also lead to p53 activation.[102,104] In a normal state, the cell

has fairly low levels of p53 because of the metabolic instability of inactivated p53.[102] In order to activate p53 after DNA damage or cellular stress, the protein must be phosphorylated.[102] A large number of kinases phosphorylate p53, including casein kinases, extracellular-signal-related kinases, JNK, protein kinase C, and Raf1 kinase.[102,105] However, the response to DNA damage is most likely mediated by DNA-PK (a caspase target), the product of the ataxia-telangiectasia gene (ATM), and the checkpoint kinase Chk2.[102,106–108] The phosphorylation state of p53 is also controlled by the action of protein phosphatases 1 and 2A, in that inhibition of these enzymes by okadaic acid leads to hyperphosphorylation of p53.[105]

Once phosphorylated, p53 then acts as a transcription factor to enhance and repress the transcription of several genes involved in apoptosis. First, p53 increases the transcription of several genes that control the redox state of the cell. The synthesis of these genes leads to the production of reactive oxygen species that subsequently cause mitochondrial apoptosis.[109] Second, p53 up-regulates the transcription of Bax and represses Bcl-2 transcription, altering the Bcl-2:Bax ratio favoring apoptosis via a mitochondrial pathway.[110,111] Third, p53 up-regulates the transcription of the Fas receptor which likely primes the cell for apoptosis induced by exposure to FasL.[103,112,113] In addition to these transcription-based mechanisms, transcription-defective p53 mutants also induce apoptosis.[114,115] This finding suggests that p53 may be able to directly signal apoptosis, however the biochemical basis for this signal is unclear.[102,111]

Table 5. Kinase pathways involved in apoptosis

Kinase	Type	Outcome
PKA	Ser/Thr	Anti-apoptotic
PKB	Ser/Thr	Anti-apoptotic
PKC	Ser/Thr	Pro- and anti-apoptotic
ERK	Ser/Thr, Tyr	Anti-apoptotic
p38 MAPK	Ser/Thr, Tyr	Pro-apoptotic
JNK	Ser/Thr, Tyr	Pro-apoptotic
Multiple	Tyr	Anti-apoptotic

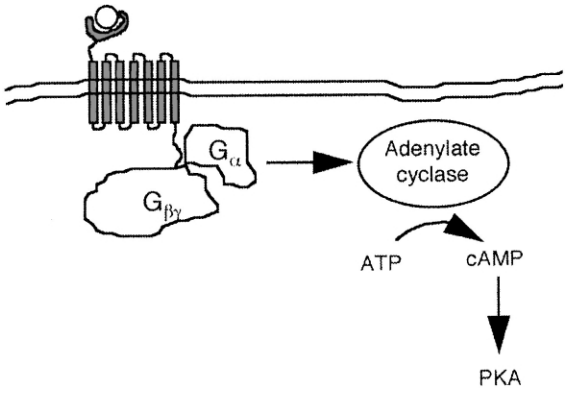

Figure 4. Activation of protein kinase A. Binding of a ligand to a seven-membered transmembrane receptor leads to the activation of Gα subunit. Activated Gα then triggers the activation of adenylate cyclase, an enzyme that converts ATP into cAMP. The increase in intracellular cAMP then activates PKA.

Kinases/phosphatases

The ability of a cell to transduce environmental signals into a biochemical or cellular response is essential for the survival of bacteria as well as multicellular organisms. In the absence of protein synthesis, one rapid means that cells use to transduce signals is through the addition or removal of a phosphate group to serine, threonine, and tyrosine amino acids. Indeed, the human genome likely contains as many as 2000 different protein kinase genes, and 1000 different protein phosphatase genes.[116] Because of the importance of phosphorylation in signal transduction, it is not surprising that the activity of protein kinases and phosphatases can modulate a cell's response to an apoptotic stimulus. Indeed, activation of protein kinases A, B, and C (PKA, PKB, PKC); the MAP kinases extracellular-signal-related kinase (ERK), JNK, and p38; and various tyrosine kinases all have been demonstrated to modulate apoptosis (Table 5).

PKA is activated by cAMP generated after ligation of a G-protein coupled surface receptor (Fig. 4).[117] Activation of protein kinase A (PKA) is generally considered to be anti-apoptotic because the addition of cAMP inhibits apoptosis induced by a variety of agents including the DNA damaging agents etoposide and camptothecin; the kinase inhibitors staurosporine, wortmannin, and LY294002; γ-irradiation; and the environmental stresses induced by heat and growth factor withdrawal.[14,118] In addition, PKA can phosphorylate and inactivate the pro-apoptotic protein Bad.[119,120]

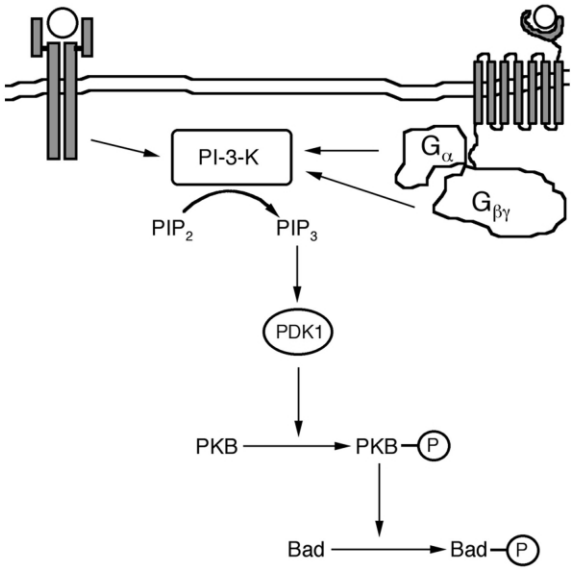

Figure 5. Protein kinase B signal transduction pathway. In the first step in this pathway, ligation of a growth factor receptor triggers activation of tyrosine kinase activity. The phosphorylated receptor recruits and activates PI-3-K. PI-3-K leads to the production of phosphorylated inositol second messengers that activate phosphoinositide-dependent protein kinase (PDK1). PDK1 phosphorylates PKB leading to its activation. Activated PKB can inhibit apoptosis by phosphorylating Bad, and subsequently preventing its interaction with Bcl-2 and Bcl-x_L.

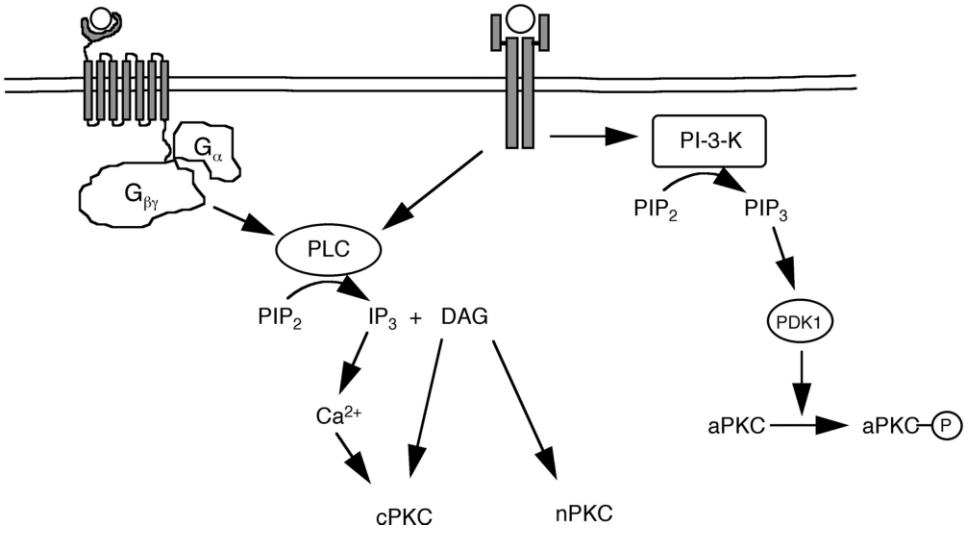

Figure 6. Activation of the three classes of protein kinase C. In this signal transduction pathway, ligation of a cell surface receptor activates an enzyme responsible for the production of lipid second messengers. The classical (cPKC) and novel PKC (nPKC) isozymes are activated following the generation of IP_3 and DAG by PLC. The atypical PKC (aPKC) isozymes can be activated via a PI-3-K-dependent production of PIP3 that activates the kinase PDK1. PDK1 can then phosphorylate and activate the aPKCs.

PKB, also known as Akt, is activated by the actions of phosphoinositol 3-kinases (PI-3-K, Fig. 5). PI-3-K is activated by both tyrosine kinases coupled to growth factor receptors and by G-protein-coupled receptors, leading to the conversion of phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-trisphosphate (PIP_3).[120,121] PIP_3 activates phosphoinositide-dependent kinase-1 (PDK1) which then phosphorylates PKB.[122,123] Activation of PKB is generally considered anti-apoptotic because it can phosphorylate and thereby inactivate the pro-apoptotic proteins Bad and caspase-9.[50,119,124,125] PKB may also inhibit apoptosis through the phosphorylation of other cellular targets including transcription factors. Furthermore, inhibition of PKB activity by the PI-3-K inhibitors wortmannin and LY294002 induces apoptosis.[125,126]

PKC refers to a large family of serine/threonine kinases that depend on phospholipids for maximum activity.[127] They are grouped into one of three categories based on their requirements for DAG and Ca^{2+}. The 'classical' PKC isoforms (α, βI, βII, γ) require both DAG and Ca^{2+}, while the 'novel' isozymes (δ, ε, η, θ) only require DAG. The 'atypical' isozymes (ζ, λ, μ) are independent of both DAG and Ca^{2+}, and can be activated by ceramides and by phosphorylation (via PDK1).[128] Activation of these kinases results from ligation of both G-protein-coupled receptors and tyrosine kinase-coupled receptors (Fig. 6).[120,129,130] Activation of these receptors activates both phospholipase C (PLC) and PI-3-K. PLC converts PIP_2 into DAG and inositol 1,4,5-triphosphate (IP_3).[131] IP_3 triggers the release of Ca^{2+} from intracellular stores. PKCs can be both pro- and anti-apoptotic. For example, PKC inhibitors induce apoptosis on their own,[126,132] and can either potentiate or inhibit apoptosis triggered by other signals.[130,133] Similarly, activation of PKCs with the phorbol ester PMA can induce lymphocyte apoptosis, but inhibit apoptosis triggered by other stimuli.[132,133]

The mitogen activated protein kinase family includes the ERK, JNK, and p38 MAP kinases. These kinases are controlled by phosphorylation on both threonine and tyrosine residues, are activated by several cascading layers of upstream kinases (Fig. 7).[117,134] Similar to PKC, the ERK pathway is activated by both growth factor associated tyrosine kinases and G-protein coupled receptors via the Ras pathway.[127,129,135] Activation of the ERK pathway is generally anti-apoptotic, as upstream members are also involved in the phosphorylation of Bad.[136,137] In contrast, the p38 and JNK

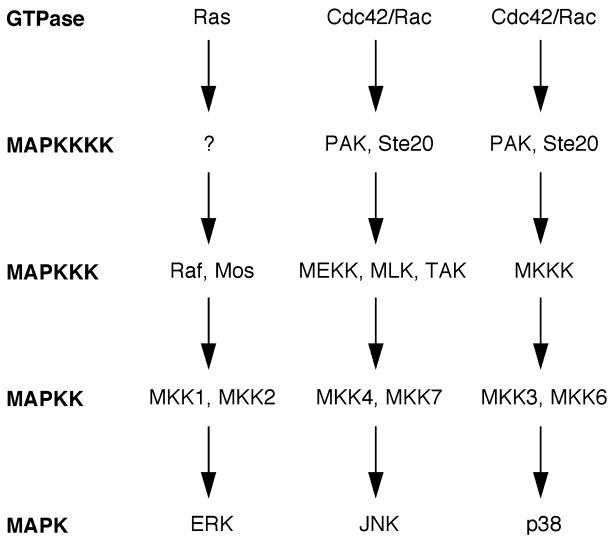

Figure 7. MAP kinase pathways. Protein kinase cascades responsible for the activation of the ERK; JNK; and p38 MAP kinases. The first step in each of these pathways is the activation of a small GTPase via the exchange of GDP for GTP. MAPKK, MAP kinase kinase; MAPKKK, MAP kinase kinase kinase; MAPKKKK, MAP kinase kinase kinase kinase; MEKK, mitogen-activated extracellular response kinase kinase; MKK, MAPK kinase; MKKK, MKK kinase; MLK, mixed lineage kinase; PAK, p21-activated kinase; TAK, Tat-associated kinase.

pathways are activated by pro-inflammatory cytokines and apoptotic stimuli including ceramides, caspases, environmental stresses, and UV-irradiation.[13,117,138] Hence, these pathways are generally considered pro-apoptotic.

In contrast to the kinase pathways discussed above, tyrosine kinases generally do not form cascading pathways with other tyrosine kinases. Instead, these kinases are associated with growth factor receptors and then feed into the ser/thr kinase cascades discussed above.[135,139] Tyrosine kinases are generally considered anti-apoptotic for several reasons. Constitutively active forms of tyrosine kinases were some of the first oncogenes discovered,[127] and overexpression of constitutively active forms can protect cells from growth factor withdrawal-induced apoptosis.[139] Tyrosine kinases typically signal via the PI-3-K and Ras pathways whereby they activate the anti-apoptotic PKB and ERK pathways.[135] However, activation of cell surface receptor-associated tyrosine kinases is not always anti-apoptotic. Ligation of the B- or T-cell receptor activates members of the Src family of tyrosine kinases.[121] Depending upon the developmental and activation state

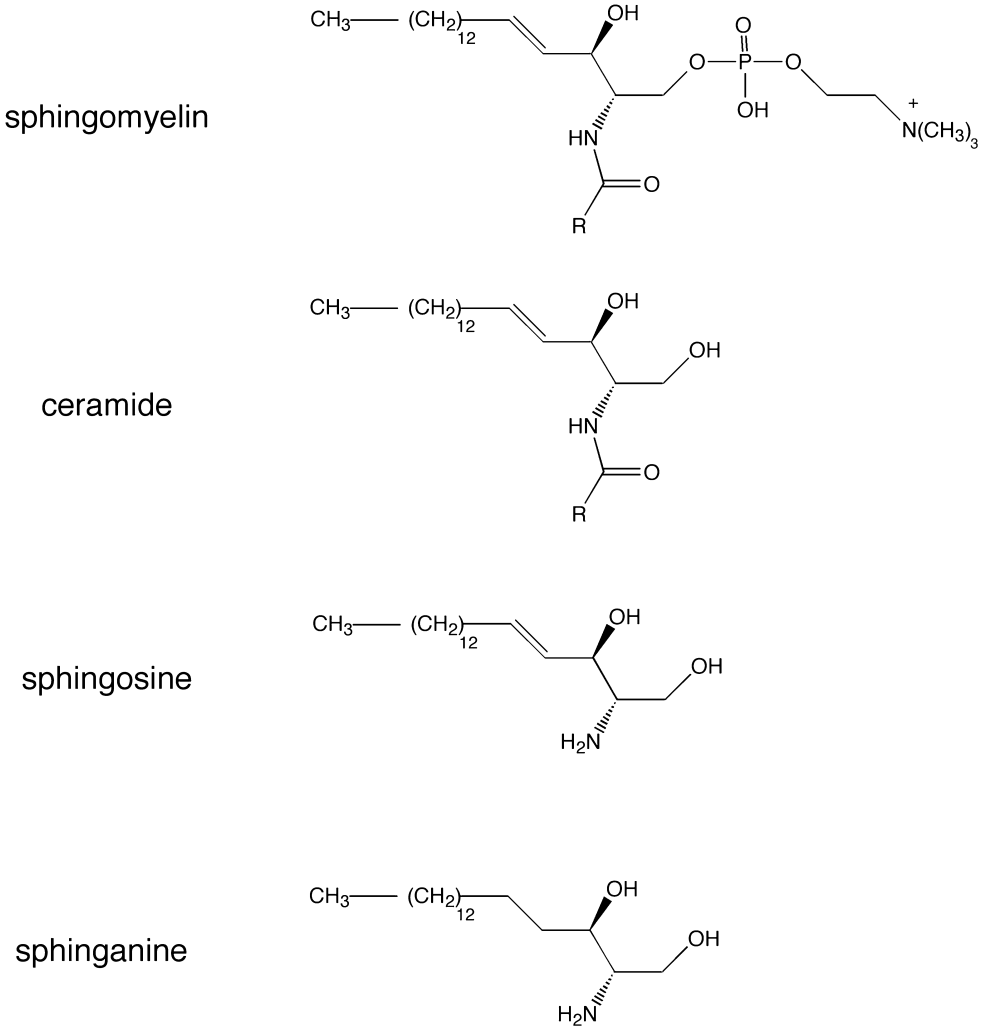

Figure 8. Chemical structures of sphingolipids.

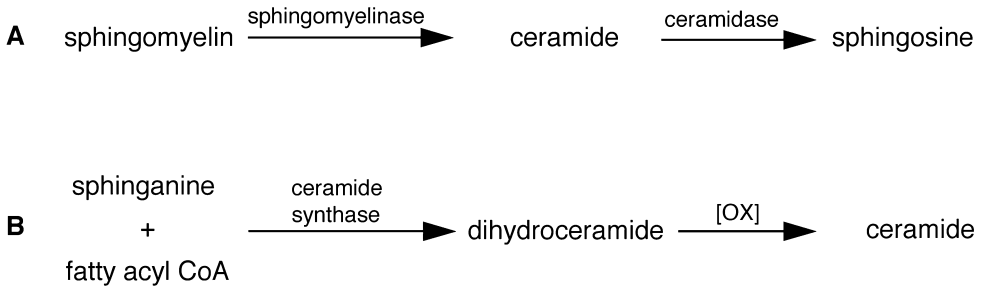

Figure 9. Pathways of ceramide formation: (A) sphingomyelinase pathway; (B) de novo pathway.

of the B- or T-cell, ligation of these receptors can induce apoptosis during negative selection in the bone marrow and thymus, or it can prevent apoptosis during positive selection that occurs during the immune response to foreign antigen.

The activity of the various kinase pathways just discussed are controlled by a variety of phosphatases.[116] In contrast to kinases which are generally inactive until activated, phosphatases generally have a relatively high level of basal activity, and hence act to turn off kinase cascades. Several major families of phosphatases that regulate apoptosis include the serine/threonine PP1 and PP2A that are inhibited by okadaic acid and calyculin A; the serine/threonine PP2B calcineurin that is inhibited by cyclosporin A and FK506; and a group of protein tyrosine phosphatases.[105,133,140,141] Similar to the kinases they regulate, these enzymes can be either pro- or anti-apoptotic depending upon the status of the cell. For example, okadaic acid can both induce apoptosis in the absence of another stimulus,[105,133,142–144] and prevent apoptosis induced by DNA damaging agents, staurosporine, and glucocorticoids.[141,145–147]

Ceramide

Ceramides belong to the class of lipid second messengers used for signal transduction that includes IP_3, PIP_3, and DAG.[148] Chemically, ceramides are composed of two long alkyl chains connected via an amide bond (Fig. 8). Ceramides can be formed by one of two processes. As a precursor during the synthesis of sphingomyelin, a component of the plasma membrane, they are formed through the condensation of sphinganine and a fatty acyl-CoA (Fig. 9).[13] In addition, through the action of specialized phospholipases known as sphingomyelinases, they are formed by the breakdown of sphingomyelin (Fig. 9).[75] The formation of ceramides is triggered by many cellular processes including the ligation of cell surface receptors, the action of chemotherapeutic drugs, reactive oxygen species, UV- and γ-radiation, and environmental stresses.[13,75,149,150] While most ceramide formation during apoptosis appears to involve the action of sphingomyelinases, there are some cases in which de novo ceramide synthesis is activated during apoptosis.[13,75,149]

Ceramides can induce apoptosis through a wide variety of mechanisms. Ceramides can activate both kinases and phosphatases including p38, JNK, ERK, ceramide-associated kinase, ceramide-associated phosphatase, and PKC ζ.[75,78,149,151–153] Ceramides inhibit PKB activity by interacting with either PI-3-K or PKB.[15,153,154] Ceramides can cause upregulation of FasL, an effect that may be mediated by its activation of JNK.[149,155] Ceramides also induce mitochondrial apoptosis. For example, ceramides have been shown to directly induce the MPT in isolated mitochondria.[94] Inside the cell, they can be converted into the ganglioside GD3 via the sequential addition of a glucose, lactose, and two sialic acid moieties to the primary hydroxyl.[156] In both whole cell and isolated mitochondrial systems, GD3 also induces the MPT.[94,156]

Conclusion

Apoptosis is a complex biochemical process that involves all aspects of the cell from the plasma membrane to the nucleus. Apoptosis stimuli are mediated by many different cellular processes including protein synthesis and degradation, the alteration in protein phosphorylation states, the activation of lipid second messenger systems, and disruption of normal mitochondrial function. Despite this diversity in signal transduction, all apoptotic pathways are believed to converge ultimately with the activation of caspases leading to the characteristic morphological changes of apoptosis.

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