

How cells die: Apoptosis pathways

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Generally speaking, there are 2 types of cell death: apoptosis and necrosis. Necrotic cell death is considered an accidental type of death, caused by gross cell injury, and results in the death of groups of cells within a tissue. In contrast, apoptotic cell death may be induced or is preprogrammed into the cell (eg, during development) and results in the death of the individual cells. Apoptotic cells may be characterized by specific morphologic and biochemical changes orchestrated by a family of cysteine proteases known as *caspases*. At the molecular level, apoptosis is tightly regulated. There are 2 main pathways to apoptotic cell death. One involves the interaction of a death receptor, such as the TNF receptor-1 or the Fas receptor with its ligand, and the second pathway depends on the participation of mitochondria. Proapoptotic and antiapoptotic members of the Bcl-2 family regulate the mitochondrial pathway. The end result of either pathway is caspase activation and the cleavage of specific cellular substrates, resulting in the morphologic and biochemical changes associated with the apoptotic phenotype. (J Allergy Clin Immunol 2001;108:S99-103.)

Key words: Apoptosis, caspases, death receptor, mitochondria

Apoptosis, or programmed cell death, is an essential physiologic process required for normal development and maintenance of tissue homeostasis. However, apoptosis is also involved in a wide range of pathologic conditions, including acute neurologic injuries, neurodegenerative diseases, cardiovascular diseases, immunological diseases, acquired immunodeficiency syndrome, and cancer. Apoptosis was first described by Kerr, Wyllie and Currie¹ in the early 1970s and is defined by the morphologic appearance of the dying cell, which includes blebbing, chromatin condensation, nuclear fragmentation, loss of adhesion and rounding (in adherent cells), and cell shrinkage. Biochemical features associated with apoptosis include high molecular weight, deoxyribonucleic acid (DNA) fragmentation and/or fragmentation into an oligonucleosomal ladder,² phosphatidyl serine externalization,³ and proteolytic cleavage of a number of intracellular substrates.⁴ As a result of the plasma membrane changes, apoptotic cells rapidly undergo phagocytosis before the release of intracellular contents and without induction of an inflammatory response.

Abbreviations used

DD:	Death domain
FADD:	Fas-associated death domain
IAP:	Inhibitor of apoptosis proteins
TNF:	Tumor necrosis factor
TNFR:	TNF receptor

THE EXECUTIONERS: CASPASES

The aspartate-specific cysteine protease (caspase) cascade is now believed to be the main pathway by which cellular death is orchestrated.^{4,5} Several different caspases are constitutively expressed in cells and reside in the cytosol in an inactive form.⁶ The most prevalent caspase in the cell is caspase-3. This caspase is ultimately responsible for the majority of the effects, although it is supported by 2 others: caspase-6 and caspase-7. Together, these 3 caspases are often referred to as the “executioner caspases” because of their role in coordinating the death of the cell. In general, caspase activation requires their cleavage, preferentially after an aspartate residue,⁷ leading to the formation of the active enzyme from the inactive proform. The caspases are believed to cause the apoptotic phenotype by cleaving or degrading several important substrates.

For example, high- and low-molecular weight DNA fragmentation is caused by the action of caspase-3 on a complex of CAD/DFF40, a nuclease, and iCAD/DFF45, its inhibitor.^{8,9} Caspase-3 cleaves the inhibitor, allowing the nuclease to cut the chromatin. Blebbing is orchestrated via the cleavage and activation of gelsolin¹⁰ and p21-activated kinase-2^{11,12} and most likely through the cleavage of fodrin,¹³ which dissociates the plasma membrane from the cytoskeleton. The result is probably an effect of microfilament tension and local release, since depolymerization of actin prevents blebbing.¹⁴ The externalization of phosphatidyl serine during apoptosis is usually caspase-dependent,¹⁵ although in some types, phosphatidyl serine externalization appears to be caspase-independent.¹⁶ The precise mechanisms have not been elucidated.

Caspases are activated to fully functional proteases by 2 cleavage events. The first proteolytic cleavage divides the chain into large and small caspase subunits, and a second cleavage removes the N-terminal prodomain. The active caspase is a tetramer of 2 large and 2 small subunits, with 2 active sites.⁶ Since proteolytic cleavage generates the mature caspases, 1 way in which these enzymes are activated is via the action of proteases, including other caspases. Thus, caspases can function in an activation cascade. “Upstream” caspases, such as caspase-8 and caspase-9, are referred to as *initiator caspases*, indicating their role in triggering apoptosis by activating the executioners.

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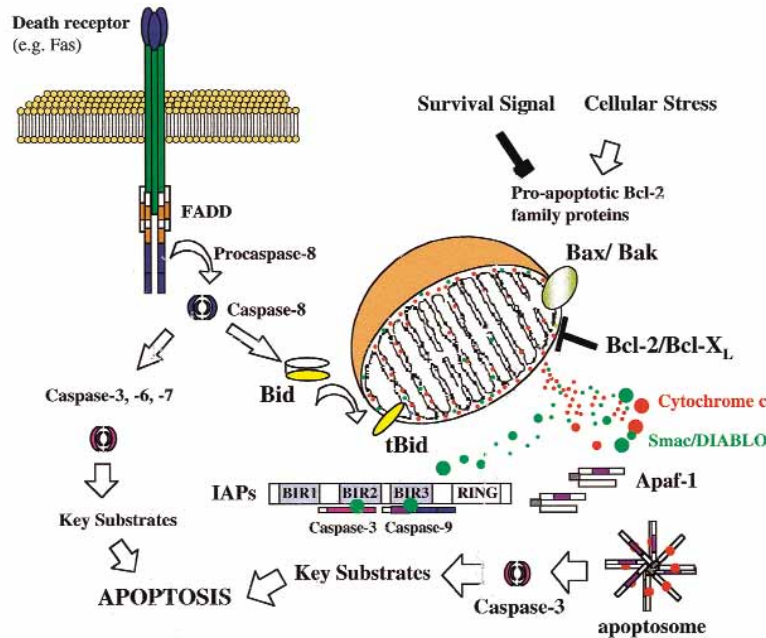


FIG 1. Apoptotic signaling pathways. Cellular stress induces proapoptotic Bcl-2 family members to translocate from the cytosol to the mitochondria, where they induce the release of cytochrome *c*. Cytochrome *c* catalyzes the oligomerization of Apaf-1, which recruits and promotes the activation of procaspase-9. This, in turn, activates procaspase-3, leading to apoptosis. Alternatively, the activation of caspase-8 by ligation of the death receptor Fas is illustrated. Ligated Fas recruits FADD to the intracellular region, which in turn recruits procaspase-8. The procaspase-8 transactivates, and the mature caspase now can cleave and activate procaspase-3, leading to apoptosis. Signaling from the Fas receptor to mitochondria involves cleavage of the BH3-only protein, Bid, by caspase-8. Bid subsequently induces cytochrome *c* release and downstream apoptotic events.

Cells also contain natural inhibitors of the caspases. These inhibitors of apoptosis proteins (IAPs) were first identified in baculovirus but were subsequently found in human cells (XIAP, c-IAP1, and c-IAP2).^{17,18} IAPs can act as direct inhibitors of the 2 death effectors, caspase-3 and caspase-7, and are able to suppress the activation of 2 initiator caspases, caspase-8 and caspase-9.¹⁸⁻²⁰ The inhibition of caspases by IAPs is mediated by domain(s) of baculovirus IAP repeats, which are present in the IAP. Baculovirus IAP repeat domains are required for binding to caspases and are necessary and sufficient for IAP function. Another region in the IAP, the RING domain, acts as a ubiquitin ligase, promoting the degradation of the IAP itself²¹ and presumably any caspase bound to it. In this fashion, IAPs put the brakes on the apoptotic process by binding; inhibiting; and perhaps, degrading caspases.

We will consider 2 general types of signaling pathways leading from a triggering event to the activation of an initiator caspase and from there to apoptotic death. The first involves death receptors such as the TNF receptor-1 and the Fas molecule, and the second one depends on the participation of mitochondria.

DEATH LIGANDS AND DEATH RECEPTORS

The death receptors of the tumor necrosis factor receptor (TNFR) family include TNFR1, Fas (CD95),

DR3/WSL, and the TRAIL/Apo-2L receptors (TRAIL-R1/DR4, TRAIL-R2/DR5). When these death receptors are bound by their ligands (TNF or lymphotoxin, Fas-ligand [FasL], the ligand for DR3, or TRAIL/Apo-2L, respectively) apoptosis can occur.

Ligation of death receptors results in the rapid formation of an intracellular death-inducing signaling complex through an amino acid stretch within the carboxy terminus of the receptor called the "death domain" (DD). This domain is responsible for coupling the death receptor to either a cascade of caspases, leading to induction of apoptosis, or to the activation of kinase-signaling pathways, resulting in gene expression through NF- κ B or activator protein 1 (AP-1). Recently, it has been suggested that there is an additional family of TNFR-related receptors that functions by sequestering the appropriate ligands. These decoy receptors do not contain the DD-signaling motif, thus they appear to operate as inhibitors of apoptosis.²²

The Fas-FasL interaction is perhaps the simplest scenario that can be used to explain the signaling pathway of death receptor-induced apoptosis. Ligation of Fas (by FasL or stimulatory antibodies) causes rapid DISC formation. The Fas-associated death domain (FADD) protein is recruited to the receptor's DD.²³ (FADD also has a DD and these domains bind via a DD-DD interaction.) FADD also contains a "death effector domain" and this, in turn, recruits procaspase-8 and binds via its death

effector domain. Procaspase-8 becomes activated via aggregation of 2 or more procaspase-8 molecules. In general, procaspase-8 has a low level of activity; however, when procaspase-8 molecules are in close proximity to one another, they can process each other to the mature, active form. The active caspase-8 then triggers the apoptotic caspase cascade.

DR3 also binds FADD and requires this adapter for induction of apoptosis.^{24,25} It is likely that DR3 signaling is similar to that of Fas. In contrast, TRAIL-receptor signaling for apoptosis can occur in the absence of FADD,²⁵ although the receptor appears to bind this adapter.²⁶ Signaling from TNFR1 is more complex than signaling through the aforementioned receptors because of the need for another adapter protein, TRADD. TRADD binds ligated TNFR1 via a DD-DD interaction. TRADD can then recruit FADD to the complex, resulting in caspase-8 recruitment and activation.

As previously mentioned, ligation of a death receptor does not necessarily lead to caspase-8 activation and death. TNFR1 can also activate NF- κ B, and in cells that are resistant to TNFR1-mediated apoptosis, inhibition of this transcription factor can sensitize the cells to this form of death.²⁷ One way NF- κ B works is by inducing the expression of 2 TNFR1-binding proteins (TRAF-1 and 2) and an IAP (c-IAP). Once bound to the receptor, the TRAFs are capable of recruiting c-IAP to the complex, and thus apoptotic signaling is inhibited.²⁸ There are most likely several other antiapoptotic functions of NF- κ B.

A molecule that is often referred to as c-FLIP but has a number of different names (ie, FLAME, I-FLICE, Casper, CASH, usurpin, MRIT, and CLARP) can block Fas-mediated apoptosis.²⁹ It resembles procaspase-8 but does not have the active proteinase site. Therefore, although it can be recruited to the ligated Fas-signaling complex, it cannot convey a death signal. Interleukin 2 (IL-2) has been shown to downregulate c-FLIP expression,³⁰ and this may explain why IL-2 can sensitize activated T cells to Fas. However, other studies have shown that c-FLIP is not recruited to the signaling complex in resistant T cells,³¹ and therefore other regulators may be important.

MITOCHONDRIA AND THE FUNCTIONS OF THE Bcl-2 FAMILY

There is accumulating evidence that the mitochondria play an essential role in many forms of apoptosis³² by releasing apoptogenic factors, such as cytochrome *c*,^{33,34} from the intermembrane space into the cytoplasm, which activates the downstream execution phase of apoptosis. In living cells, mitochondrial changes are predominantly prevented by antiapoptotic members of the Bcl-2 family of proteins. Bcl-2 was first discovered as a proto-oncogene in follicular B-cell lymphoma. Subsequently, it was identified as a mammalian homolog to the apoptosis repressor *ced-9* in *Caenorhabditis elegans*. Since then, at least 19 Bcl-2 family members have been identified in mammalian cells, which possess at least 1 of 4 conserved

motifs known as *Bcl-2* homology domains (*BH1* to *BH4*). The Bcl-2 family members can be subdivided into 3 categories according to their function and structure: antiapoptotic members, such as Bcl-2, Bcl-XL, Bcl-w, Mc1-1, and A1 (Bf1-1); proapoptotic molecules, such as Bax, Bak, and Bok (Mtd); and the BH3-only proteins, Bid, Bad, and Bim (these are called BH3-only proteins because of 4 Bcl-2 homology regions, they share only the third).

Subcellular localization studies have shown that Bcl-2 and Bcl-XL reside on the mitochondrial outer membrane, whereas the proapoptotic family members may either be cytosolic or present on the mitochondrial membrane. Although the proapoptotic Bcl-2 family members are also found elsewhere (eg, endoplasmic reticulum, nuclear envelope), their major effect appears to be on the mitochondria, apparently undergoing a conformational change³⁵ that possibly leads to the exposure of the proapoptotic BH3 domain through several mechanisms, including dephosphorylation (eg, Bad)³⁶ and proteolytic cleavage by caspases (eg, Bid).³⁷ Bax translocation to the mitochondria involves homo-oligomerization.³⁸ Translocation of Bax, Bid, or Bad can induce a dramatic event in the mitochondria. These organelles then release the proteins contained in the intermembrane space, including 1 key protein, cytochrome *c*.

Cytochrome *c* is encoded by a nuclear gene, but when it is imported into the mitochondria, it is coupled with a heme group to become holocytochrome *c*, and it is only this form that induces caspase activation.³⁴

While the antiapoptotic proteins Bcl-2 and Bcl-XL work to prevent cytochrome *c* release from mitochondria, thereby preserving cell survival,^{34,33} the proapoptotic members play a major role in initiating cytochrome *c* release. For example, Bid is implicated in Fas-mediated apoptosis, Bax in DNA damage-induced apoptosis and neurotrophin deprivation-induced death, and Bad in lymphokine deprivation-induced cell death in certain neuronal cells.

When cells release cytochrome *c*, they do so suddenly, completely, and apparently from all of the mitochondria in the cell (this has been established with only 1 cell line, but it may be generally true).³⁹ After cytochrome *c* release, caspases are activated, and the cells undergo apoptosis. This occurs through the formation of an "apoptosome" (consisting of cytochrome *c*, apoptotic protease activating factor -1 [Apaf-1], and procaspase-9) and is dependent on both cytochrome *c* from the mitochondria and either ATP or dATP in the cell. Apaf-1^{40,41} is a protein contained in the cytosol, and cytochrome *c* binds and induces it to oligomerize. This then recruits an initiator caspase, procaspase-9.^{41,42} Unlike other caspases, procaspase-9 does not appear to be activated simply by cleavage, but instead must be bound to Apaf-1 to be active.^{43,44} These proteins interact via CARD-CARD (caspase recruitment domain) interactions. The apoptosome now recruits procaspase-3, which is cleaved and activated by the active caspase-9 and released to mediate apoptosis.

Recently, a new protein with the dual name Smac/DIABLO^{45,46} has been discovered, which is also released from the mitochondria along with cytochrome *c*

during apoptosis. This protein promotes caspase activation by associating with the apoptosome and inhibiting IAPs. It has been shown that cytochrome *c* and Smac/DIABLO are released coordinately. While cytochrome *c* activates Apaf-1, Smac/DIABLO relieves the inhibition on caspases by IAPs, and apoptosis can proceed. In some cellular systems, cytochrome *c* is necessary, but not sufficient, for cell death. In these systems, Smac/DIABLO may be the second factor required for the so-called competence to die.⁴⁷⁻⁴⁹

DEATH RECEPTORS AND MITOCHONDRIA

The mitochondrial pathway involving Bcl-2 family members, mitochondria, cytochrome *c*, Apaf-1, and caspase-9 is fundamentally distinct from that of the death receptors. Cells lacking caspase-8⁵⁰ or FADD²⁵ do not respond to death ligands but are capable of undergoing apoptosis that is induced by other agents, such as cellular stress. Cells lacking caspase-9^{51,52} or Apaf-1^{53,54} are incapable of undergoing apoptosis that is induced by such stressors, but they readily die in response to death ligands.

A controversy arises, however, when we consider the antiapoptotic effects of Bcl-2 on signaling by Fas. Some investigators have found that Bcl-2 can prevent Fas-mediated apoptosis.⁵⁵ However, others contend that Bcl-2 family members never interfere with Fas-mediated apoptosis.⁵⁶ However, if Bcl-2 can sometimes interfere with death mediated through Fas, is this through a new function of the antiapoptotic protein or a role of the mitochondria?

Evidence suggests the latter. One of the BH3-only proteins, Bid, is cleaved by active caspase-8 (activated by ligation of the death receptors) and translocates to the mitochondria, where it triggers cytochrome *c* release,^{37,57,58} most likely through interaction with Bax^{35,59} or Bak. Bcl-2 can prevent this Bid-mediated effect. In cells, expression of Bid can sensitize for TNF-induced apoptosis.⁵⁷ In vitro, the ability of caspase-8 to activate caspase-3 is greatly facilitated by the presence of Bid and mitochondria.⁶⁰ These observations suggest that when caspase-8 is limiting, the action of Bid on mitochondria can determine whether a cell will undergo apoptosis (via cytochrome *c* release and the activation of caspase-9). This is supported by the finding that Bid-deficient mice are relatively resistant to the lethal effects of anti-Fas antibody in vivo, and their hepatocytes do not readily undergo Fas-mediated death.⁶¹ In contrast, lymphoid cells from these mice are fully sensitive to death via Fas ligation.

SUMMARY

The apoptotic pathways we have briefly outlined here are the only pathways for apoptosis that have been unambiguously defined. Are there others? Almost certainly, but at this point we do not know how they are related to cell death or how their activities might overlap with those of the death receptors or mitochondrial pathways. Furthermore, it is becoming increasingly clear that in many cases, active cell death that is controlled at the molecular

level can occur even in the absence of caspase activation. These notions and observations present important challenges to our concepts of apoptotic pathways and lead us into new areas in our search to understand how cells die.

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