

# Apoptosis as a Mechanism of Cell Control

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## Introduction

With the possible exception of cancer genetics, no topic in the biological sciences has generated as much interest and experimentation over the past decade as the topic of apoptosis. The widespread interest is best shown by the outbreak of papers dealing with apoptosis or programmed cell death—over 5,000 papers in the last 5 years—whereas relative few papers covered the topic during the period after apoptosis was initially described.

Unlike necrosis, apoptosis is an active cellular process involving genes, enzyme activation, signaling, and DNA fragmentation. Recent studies have revealed that most of the genes and proteins that are needed to operate in apoptosis do exist in cells. Hence, apoptosis is often called cell suicide in the sense that cells are dying via apoptosis by activating a preexisting machinery for suicide. Apoptotic cells do not induce an inflammatory response but are targets for phagocytosis by adjacent macrophages.

## Overview

A variety of stimuli act as triggers leading to the onset of the apoptotic program through modulating substances, such as cytokines, genes, adapters, and interacting proteins. Modulators represent most of the work in the field in understanding how key pro- and anti-apoptotic proteins interact in transduction of the signal and determination of the cellular outcome of life or death. Effectors are ubiquitous molecules present in every cell that are activated in the event a cell chooses to die. To date, the only definitely identified effectors are cysteine proteases of the caspase family. Caspases are present as inactive proenzymes, which are activated by proteolysis, then cleave and activate each other in a cascade-like fashion. By their cleavage of a variety of intracellular cytoskeletal, nuclear, and cytosolic proteins, most of the morphological and biochemical events associated with apoptosis emerge.

Apoptosis is an evolutionarily conserved process. The nematode *Caenorhabditis elegans* has provided the best model for studying the core elements of apoptosis. Three gene products are essential in nematode apoptosis: CED-3 and CED-4 promote apoptosis, while CED-9 inhibits apoptosis. CED-3 itself is a caspase, a cysteine protease that cleaves certain proteins after specific aspartic acid residues; it exists as a proenzyme, which is activated through self cleavage. CED-4 binds to CED-3 and promotes CED-3 activation, whereas CED-9 binds to CED-4 and prevents it from activating CED-3. Normally, CED-9 is forming an inactive ternary complex of the three proteins called apoptosomes, keeping CED-3 inactive<sup>[1]</sup>. Apoptotic stimuli cause CED-9 dissociation, allowing CED-3 activation and

thereby committing the nematode cell to die by apoptosis. Vertebrates have evolved entire gene families resembling nematode cell death genes. Apaf-1 is the only mammalian CED-4 homolog known to date. The mammalian Bcl-2 gene family are related to CED-9, but include two subgroups of proteins that either inhibit or promote apoptosis.

## Basic Apoptosis Machinery

In dying nematodes, the BH3 domain protein EGL-1 dislodges CED-4/CED-3 from its CED-9 anchor, leading to oligomerization of the complex, processing and activation of CED-3 and programmed cell death<sup>[2]</sup>. Apaf-1, the mammalian homolog of CED-4, interacts with both Bcl-2 and caspase-9 (homologs of CED-9 and CED-3, respectively), and promotes caspase activation<sup>[3]</sup>. Apaf-1 itself is Bcl-2 binds Apaf-1 and prevents caspase-9 activation. A death signal provokes interaction of a BH3 family member (Bik or regulated by the Bcl-2 family. Bax) with Bcl-2, preventing it from neutralizing Apaf-1. In the presence of cytochrome c released from mitochondria and ATP, Apaf-1 can then bind to procaspase-9 through caspase recruitment domain (CARD) and promote its dimerization and activation.<sup>[3]</sup> Caspase-9 subsequently activates effector caspases. Thus, the CED-9/bcl-2 family integrates positive and negative signals and arbitrates whether apoptosis should occur; activation of CED-4/Apaf-1 commits to apoptosis, and CED-3/caspase mediates the death process.

In mammalian cells, the Bcl-2 family likewise rules on signals from diverse cytotoxic stimuli (e.g. cytokine deprivation and exposure to glucocorticoids, DNA damage, or staurosporine)<sup>[4]</sup>. However, the signal induced by the death receptor CD95/Fas proceeds primarily through the adapter, Fas-associated death domain (FADD), which directly activates caspase-8 and largely bypasses the Bcl-2 family<sup>[5]</sup>. Clustering of the receptors' intracytoplasmic death domain (DD), subsequent binding of FADD, and its binding to procaspase-8 (FLICE) through a "death effector domain" (DED) which functions as a CARD, represent the sequence of events that follow subsequently<sup>[5]</sup>.

## Opposing Roles of the Bcl-2 Oncogene Family

At least 15 Bcl-2 family members have been identified in mammalian cells. All members possess at least one of four conserved motifs known as Bcl-2 homology domains, BH1 to BH4<sup>[6]</sup>. The anti-apoptotic members of this family include Bcl-2, Bcl-XL, Mcl-1. Conversely, the pro-apoptotic, death-promoting members include Bax, Bcl-Xs, Bad, Bak, Bik, and Bid. BH3 domain proteins represent the physiologic antagonists of the pro-survival proteins. BH3 is essential for the function of the "killers", including EGL-1.

Pro- and anti-apoptotic family members can heterodimerize and seemingly titrate one another's function, suggesting that their relative concentrations may act as a rheostat for the suicidal program. BH1, BH2, and BH3 domains strongly influence homo- and hetero-dimerization and the three-dimensional structure of Bcl-X<sub>L</sub>. Heterodimerization is not required for pro-survival function. For pro-apoptotic activity, heterodimerization is essential in the BH3 domain group. Bcl-X<sub>L</sub> binds to the CED-4-like portion of Apaf-1, whereas procaspase-9 binds to its NH2-terminal CARD. Bcl-X<sub>L</sub> may inhibit the association of Apaf-1 with procaspase-9 and thereby prevent caspase-9 activation. Pro-apoptotic relatives like Bik may free CED-4/Apaf-1 from the death inhibitor<sup>[6]</sup>.

### Signaling by CD95 and TNF

Death receptors—cell surface receptors that transmit apoptosis signals initiated by specific “death ligands”—play a central role in instructive apoptosis<sup>[5]</sup>. These receptors can activate death caspases within seconds of ligand binding, causing the apoptotic demise of the cell within hours. Death receptors belong to the TNF receptor superfamily, which is defined by similar cysteine-rich extracellular domains. The death receptors contain in addition a homologous cytoplasmic sequence termed as the DD. The best characterized death receptors are CD95 (Fas or Apo1) and TNFR1. CD95 ligand (CD95L) binds to CD95; TNF and lymphotoxin bind to TNFR1. CD95 and CD95L play an important role mainly in three types of physiologic apoptosis: peripheral deletion of activated mature T cells at the end of an immune response; killing of targets such as virus-infected cells or cancer cells by cytotoxic T cells and by NK cells; and killing of inflammatory cells at immune privileged sites such as the eye<sup>[1]</sup>.

As discussed already, the signaling pathway induced by CD95/Fas bypasses the regulation by Bcl-2 family<sup>[5,6]</sup>. Because death domains have a propensity to associate with one another, CD95 ligation leads to clustering of the receptors' death domain. An adapter protein called FADD, also called Mort 1, then binds through its own death domain to the clustered receptor death domains. FADD also contains a DED that binds to analogous domain repeated in tandem within the zymogen form of caspase-8 (also called FLICE or MACH). The DED is a specific example of a more global homophilic interaction domain termed CARD, also found in other caspases including caspases-2, -8, -9, and -10. Upon recruitment by FADD, caspase-8 oligomerization drives its activation through self-cleavage. Caspase-8 then activates downstream effector caspases such as caspase-9, the mammalian functional homolog of CED-3, committing the cell to apoptosis.

TNF is produced mainly by activated macrophages and T cells in response to infection. By engaging TNFR1, TNF activates the transcription factors NF-κB and AP-1. In some cell types, TNF also induces apoptosis through TNFR1. Unlike CD95L, however, TNF rarely triggers apoptosis unless protein synthesis is blocked, which suggests the preex-

istence of cellular factors that can suppress the apoptotic stimulus generated by TNF. Expression of these suppressive proteins is probably controlled through NF-κB and JNK/AP-1, as inhibitors of either pathway sensitizes cells to apoptotic induction by TNF. NF-κB appears to activate a group of gene products that function cooperatively at the early checkpoint to suppress TNF-mediated apoptosis) and that function more distally to suppress genotoxic agent-mediated apoptosis<sup>[7]</sup>.

### Effector phase of apoptosis; caspases

The effector component of the apoptotic machinery is a proteolytic system involving a family of proteases, the caspases. Apoptotic events such as DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinkage, and disassembly into membrane-enclosed vesicles (apoptotic bodies) all result from the cleavage of certain cellular proteins by caspases. For example, caspases contribute to apoptosis through direct disassembly of cell structures, as illustrated by the destruction of nuclear lamina (a structure involved in chromatin organization). During apoptosis, lamina is cleaved at a single site by caspases, causing lamina to collapse and contributing to chromatin condensation. Caspases also recognize cell structures indirectly by cleaving several proteins involved in cytoskeleton regulation, including gelsolin, focal adhesion kinase (FAK), and p21-activated kinase 2 (PAK2). Given the function of caspases as mediators of cell death, the complexity of their regulation is likely to rival that of the coagulation and complement systems<sup>[8]</sup>.

Caspases are all expressed as proenzymes that contain three domains: an amino terminal domain, a large subunit and a small subunit<sup>[9]</sup>. There are at least 13 caspases. Activation involves proteolytic processing between domains, followed by association of the large and small subunits to form a heterodimer<sup>[9]</sup>. Caspases are specific proteases, with an unusual and absolute requirement for cleavage after aspartic acid. Initiator caspases are involved in upstream regulatory events, while effector caspases are responsible for proteolytic cleavages that lead to cell disassembly. Effector caspases (caspase-6, -3, -7) are activated by different initiator caspases (caspase-9, -8, -10), each of which is activated by a set of proapoptotic signals. One role of the caspases is to inactivate proteins that protect living cells from apoptosis. A clear example is the cleavage of ICAD/DEF45, an inhibitor of the nuclease responsible for DNA fragmentation, caspase-activated deoxyribonuclease (CAD). In nonapoptotic cells, CAD is present as an inactive complex with ICAD. During apoptosis, ICAD is inactivated by caspases, leaving CAD free to function as a nuclease<sup>[8]</sup>.

Caspases can be activated by two distinct mechanisms. One is the mutual cascade-like activation following exposure of the cell to a previously activated caspase molecule. This cascade is then used extensively by the cell for the activation of the downstream caspases: caspases -3, -6, and -7. The second strategy, “induced proximity,” was first described in caspase-8, an initiator caspase that acts down-

stream of the Fas/CD95 death receptor. Upon ligand binding, Fas receptor molecules aggregate into a membrane-bound complex. This signaling complex recruits, via the receptor-bound adapter protein FADD, several procaspase-8 molecules, resulting in a high local concentration of procaspase-8, as described above<sup>[5,6]</sup>.

Caspases are regulated by opposing effects of activators and inhibitors. A signal apparently initiates three pathways involving cofactors, initiator caspases, and inhibitors. Activation of cofactors, such as cytochrome c release from mitochondria to cytoplasm, modification of the caspase, such as relocation of caspase 8 to a receptor complex; and inactivation of inhibitors together result in activation of the initiator caspase. Examples of caspase inhibitors include FADD-like ICE inhibitory proteins (FLIP), apoptosis repressor with caspase recruitment domain (ARC), and a family of apoptosis inhibitors (IAPs)<sup>[8]</sup>.

### Apoptotic and Antiapoptotic Genes and Signals

Anti-apoptotic (prosurvival) signals include hematopoietic growth factors (HGF) including Tpo<sup>[10]</sup>, and genes such as Bcl-2, Bcl-X, and ras. Pro-apoptotic (death) signals include HGFs withdrawal, negative cytokines (TNF, TGF, IFN), genes (Bad, Bax, Bak; Fas/Fas-L system, and myc). Wild-type p53 is a known apoptotic gene, while mutated p53 or p53 inactivator Mdm2 (the human analog of the mouse double minute-2, binds and abrogates p53 activity)<sup>[11]</sup>. Certain cell cycle regulators are also related to cell survival or cell death.

P53 is well known as the transducer of a variety of apoptotic signals. However, apoptosis may occur via p53-dependent or independent means<sup>[12,13]</sup>. Oncoprotein-induced apoptosis may merely reflect the fact that the machinery mediating growth and apoptosis are coupled processes. In this model, apoptosis is not caused by any conflict. Rather, activation of cell proliferation necessarily primes the cellular apoptotic program that, unless countermanded by appropriate survival signals, automatically removes the affected cells. Survival signals are normally provided by neighboring cells. Thus, it is the balance between pro-apoptotic growth processes and anti-apoptotic survival signals that determines whether a cell proliferates or dies. The same applies to c-myc, which also acts as a potent inducer of apoptosis for hematopoietic and fibroblastic cells. Since c-myc has normally been associated with cell proliferation, it becomes clear that this gene can induce signals for proliferation versus death, depending on environmental cues, such as the presence or absence of growth factor stimuli or the relative levels of Bcl-2 family members.

### Apoptosis in Human Physiology

Apoptosis has been implicated in a variety of physiologic conditions. These range from the developmentally regulated death of different cells during normal embryonic development to Fas-induced cell death of activated lymphocytes at the completion of the immune response. In steady state hematopoiesis, blood cells undergo constant renewal from

hematopoietic stem cells. The control of cell number of each cell lineage is determined by a delicate balance between cell proliferation and cell death. In addition to stimulating the proliferation of hematopoietic progenitor cells, HGFs are required to support the survival of their target cells. If deprived of growth factors, hematopoietic progenitor cells rapidly undergo apoptosis *in vitro*. HGFs are also important in regulating the survival of mature blood cells such as neutrophils.

### Apoptosis and Diseases

In addition to its physiologic roles, apoptosis has also been implicated in certain pathological conditions. An increase or decrease of blood cells can often be explained by a decrease or an increase in apoptosis of the immature cells belonging to the respective cell lineages. Excessive apoptosis appears to play an important pathogenetic role in ineffective hematopoiesis, such as thalassemia, myelodysplastic syndromes and folate deficiency, as well as in bone marrow failures such as aplastic anemia. Conversely, the failure of cells to undergo apoptosis at a normal rate most likely contributes to the pathogenesis of many hematological malignancies<sup>[14]</sup>. Therapeutic manipulations of apoptosis will be undoubtedly one of the most important goals in these clinical settings.

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