General View of the Cytoplasmic and Nuclear Features of Apoptosis

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Abbreviations

AIF apoptosis-inducing factor
Apaf-1 apoptotic protease activating factor-1
ATP adenosine triphosphate
BA bongkrekic acid
Bcl-2 B-cell lymphoma-2
BID BH3-interacting domain death agonist
bp base pair
CAD caspase-activated DNase
c-FLIP cellular FLICE-inhibitory protein
Chx cyclohexamide
CsA cyclosporine A
CTL cytotoxic T lymphocyte
cyt c cytochrome c
DISC death-inducing signal complex
DED death effector domain
endo D endonuclease D
endo G endonuclease G
ER endoplasmic reticulum
FADD Fas-associated death-domain protein
FasL fatty acid synthetase ligand
FasR fatty acid synthetase receptor
Gzm-A granzyme-A
Gzm-B granzyme-B
ICAD inhibitor of caspase-activated DNase
Kb kilobase
MEF mouse embryonic fibroblast
MOMP mitochondrial outer-membrane permeabilization
NK natural killer
OMM outer mitochondrial membrane
PCD programmed cell death
PFN Perforin
1.1 Introduction

The normal development of a cell and the life cycles of the multicellular organism rely on a finely tuned balance between cell survival and death. In a biological context, cells need to grow, divide, and die. In regard to the latter process, cells have developed a very precisely regulated means of programmed cell death (PCD), which contributes to the maintenance of normal cell turnover, leading to reduced impact on tissues, organs, and the organism itself. Some cells have evolved a PCD process called apoptosis. Apoptosis can be simply defined as a set of biochemical cytoplasmic and mitochondrial events that may lead to the execution phase of nuclear events.

A wide array of stress stimuli can trigger the apoptotic process, and the biochemical signal can then be amplified in the cytoplasm and mitochondria by both extrinsic and intrinsic pathways. The convergence of the apoptotic signal is considered the activation of a family of cysteine aspartyl-specific proteases (caspases), composed of 12 proteins strictly involved in the apoptotic cell death process. The dying cells activate the execution pathway that leads to the appearance of blebs and to the “pinching off” of many of them, forming “apoptotic bodies,” which may be rounded and retracted from their own tissue. Subsequently, the immune system cells are able to eliminate the apoptotic bodies through an engulfment cell process. The morphological and biochemical features during the apoptotic process are not fully understood.

At the nuclear level, it is well established that endonucleases and exonucleases may hydrolyze the DNA into small fragments (200 pb) [1]. The nuclear events depend on caspase activation. Caspase 3 is considered the most important protease of the executioner pathway, and is activated by different initiator caspases. For instance, caspase 8 is activated from the death receptor, caspase 9 is involved in the mitochondrial apoptotic process, and caspase 10 is involved in the Perforin/granzyme (PFN/Gzm) pathways. The cleaved caspase 3 cleaves the endonuclease caspase-activated DNase (CAD), degrading the DNA at nucleosomal linkers [2,3], which generates small DNA fragments (∼50–300 kb). The subsequent processing of the DNA by exonucleases and endonucleases leads to the formation of 200 bp fragments. Many organelles, such as the Golgi apparatus, endoplasmic reticulum (ER), lysosomes, and mitochondria, can be recycled or eliminated, depending on the apoptotic stimuli. It is important to note that mitochondria play a pivotal role in apoptosis, since they can release cytochrome c (cyt c) and endonuclease D (endo D), leading to cell death [4,5].

One of the apoptotic pathways is the extrinsic or death-receptor pathway. It depends for its activation on a death domain and a death ligand, such as tumor necrosis factor...
alpha (TNFα) and tumor necrosis factor receptor 1 (TNFR1). The ligand represents the external death signal, leading to the intracellular signaling of the effector pathway. The main receptors recruit adaptor proteins like Fas-associated death-domain protein (FADD), TNF receptor-associated death domain (TRADD), and receptor-interacting protein (RIP) [6–8], which in turn recruit other molecules such as pro-caspase 8. The dimerization of the death effector domain (DED) leads to the formation of a death-inducing signal complex (DISC), triggering the subsequent process of autocatalysis of pro-caspase 8 to an activated protein (caspase 8) [9]. Caspase 8 activation is considered the main feature that starts the extrinsic pathway, leading to cell death. In many cases, depending on the apoptotic stimuli, the extrinsic pathway can crosstalk with the intrinsic pathway through proteolysis of the BH3-only protein, BH3-interacting domain death agonist (BID), which is what promotes the release of cyt c from the mitochondria into the cytoplasm. In the cytoplasm, cyt c may be assembled with the adaptor protein apoptotic protease activating factor-1 (Apaf-1) and ATP, generating in the cytosol the multi-molecular holoenzyme complex called the “apoptosome” (Figure 1.1) [10].

The PFN/Gzm pathway is considered part of the extrinsic pathway. It is activated when cells are infected by viruses and/or bacteria. Mechanistically, cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells produce granzymes. The granzymes, together with the PFN, may facilitate the pore formation and action of Gzms that lead to cell death. Two types of Gzm, type A (GmzA) and type B (GmzB), have been described. GmzA is considered the most important serine-protease present in CTL and NK granules. GmzB relies on the mechanism of oligomerization of PFN to enter into the cell, and depends for its action on the activation of caspases, principally caspase 3 [11,12].

It is important to note that different stimuli and players are enlisted in the various apoptotic processes. Moreover, it is well known that immune-system cells, mitochondria, and nuclear events are involved in apoptosis. The entire biochemical process is still elusive. Accordingly, the current chapter will focus on cytoplasmic and nuclear events. We describe and highlight an overview of cytoplasmic events, including the extrinsic pathway and perforin/granzyme pathways (GmzA and GmzB), as well as the main nuclear features of the current process, by correlating these events with the intrinsic apoptotic pathway. We then use such pathway characteristics to explore in more detail the mechanisms of the regulation of important human diseases, including cancer and neurodegenerative diseases.

### 1.2 Cytoplasmic Events

During apoptosis, the dying cells become rounded and retracted from the tissue. Defined “blebs” appear within the cells. The process culminates in the “pinching off” of many blebs, producing “apoptotic bodies.” These bodies recruit phagocytes, which engulf them to recycle some of the molecules. The immune system represents the major mechanism capable of eliminating the apoptotic bodies, apparently in the same way that phagocytes eliminate “non-self” particles. These morphological and biochemical features of apoptosis have been extensively studied, but the whole mechanism is not fully understood.

It is well established that during the apoptotic process, the nuclear DNA is condensed and fragmented. Several proteins, such as endonucleases and exonucleases, may degrade the DNA chain by hydrolyzing it in small fragments, with approximately 200 bp [1]. In
the cytoplasm, the Golgi apparatus, ER, lysosomes, and mitochondria can be eliminated or recycled, depending on the apoptotic stimuli. For instance, during oxidative stress involving an increase of reactive oxygen species (ROS), several mitochondrial proteins, such as cyt c and endo D, may be released from the mitochondrial intermembrane space into the cytoplasm and nucleus, leading to cell death [4,5]. Interestingly, it has been identified that the mitochondria, ER, and nucleus are targets of the Grzm pathway, which may trigger apoptosis, facilitating the PCD process.

1.2.1 The Extrinsic Pathway

The extrinsic pathway, also called the death receptor pathway, is involved in transmembrane receptor-mediated interactions, including those of the TNF family [13],
which shares the features of the cysteine-rich extracellular domains and a cytoplasmic domain (death domain) [14]. The death ligand represents the external death signal from the cell surface to the intracellular signaling and effector pathways. The mechanism requires the binding of the extracellular death ligands to the transmembrane cell receptors. The best-characterized ligands and their corresponding death receptors have been identified: (i) TNFα and TNFR1; (ii) fatty acid synthetase ligand and fatty acid synthetase receptor (FasL and FasR); (iii) Apo2 ligand and death receptor 4 (Apo2L and DR4); (iv) Apo2 ligand and death receptor 5 (Apo2L and DR5); and (v) Apo3 ligand and death receptor 3 (Apo3L and DR3) [14–18]. The receptors form clusters and can bind with their cognate trimeric ligands, leading to the recruitment of adaptor proteins, including FADD, TRADD, and RIP [6–8]. In turn, FADD or TRADD can recruit several molecules, such as pro-caspase 8, binding to them via dimerization of the DED, leading to DISC formation and subsequent autocatalysis of pro-caspase 8 and its active form (caspase 8) [9]. Caspase 8 activation is considered the main feature that triggers the extrinsic pathway, leading to cell death. Activated caspase 8 is involved in many proteolytic processes, including the activation of caspases 3, caspase 6, and caspase 7. These enzymes help induce the execution phase of apoptosis (Figure 1.1).

Depending on the apoptotic stimuli, the extrinsic pathway can crosstalk with the intrinsic pathway through proteolysis of the BH3-only protein, BID. The truncated BID (tBID) protein promotes release of mitochondrial cyt c into the cytoplasm, where it can assemble with the apoptosome complex, leading to cell death [10]. However, death receptor-mediated apoptosis can be inhibited by cellular FLICE-inhibitory protein (c-FLIP), which binds to both FADD and caspase 8, inactivating the autocatalytic effect of the caspase 8 complex [19,20]. Different mechanisms of inhibition of the extrinsic apoptosis pathway have also been described, including via the protein Toso, which blocks Fas-induced apoptosis, inhibiting the processing of caspase 8 in immune cells [21].

### 1.2.1.1 The Perforin/Granzyme Pathway

To eliminate potential dangerous cells like tumor cells or cells infected by viruses or bacteria, the immune system relies on CTLs and NK cells, both of which are produced by the action of Gzms. PFN, a protein capable of binding the membrane of the target cell, facilitate the pore formation that permits the action of Gzms. Gzms are considered specific serine-proteases involved in cell death. They are produced as inactive precursor molecules, designed to avoid the self-destruction of CTLs and NK cells. In human cells, five different Gzms have been reported. GzmA and GzmK are located on chromosome 5 and act as tryptases that cleave proteins following arginine or lysine (basic) residues. GzmB and GzmH are located on chromosome 14. GzmM is located on chromosome 19 and cleaves following methionine or leucine basic residues [22]. There are two Gzm-dependent pathways involved in cell death.

#### 1.2.1.1.1 The Granzyme A Pathway

The GzmA is considered the most important serine-protease mechanism described in CTL and NK granules. Unlike the GzmB pathway, which relies on the oligomerization of the PFN to enter into the target cell, the GzmA can activate a parallel pathway in a caspase-independent manner, leading to DNA degradation, such as single-stranded DNA damage [23]. Intracellular GzmA substrates have been found in the cytoplasm (Pro-IL-1β) [24], mitochondria (NDUFS3) [25], ER, and nucleus (SET1, APE1, HMGB2) [26–29], and are associated with histone H1, core histones, lamin A, B,
and C, Ku70, and PARP1 [30–33]. Various stimuli can trigger the GzmA pathway, such as ROS generation, the loss of membrane potential, and mitochondrial swelling. This can lead to the disruption of the nuclear envelope, inhibition of DNA repair, and activation of cytokines, as a consequence of the accumulation of GzmA in the nucleus [34]. Between mitochondrial changes (within minutes) and phosphatidyl serine externalization (30 minutes to 1 hour), dying cells can recruit the macrophage scavenger system [23].

GzmA is less cytotoxic than the GzmB pathway, which is active at micromolar-range concentrations [35]. At 2 hours after the stimulation of apoptosis, several features are present. This cell-death pathway does not activate caspases, because cell-death GzmA activation is known as a non-apoptotic death [36]. Moreover, GzmA does not permeabilize the outer mitochondrial membrane (OMM), avoiding the releasing of mitochondrial apoptotic mediators like cyt c. The entry of GzmA into the mitochondria can be partially inhibited by cyclosporine A (CsA) and bongkrekic acid (BA), suggesting a role of permeability for the transition pore (PT) in GzmA mitochondrial damage [23,35].

The oxidative damage drives the ER to make the ER-associated oxidative stress response complex (SET), which contains two endonucleases (Ape1 and NM23-H1) and a 5′-3′ exonuclease (Trex1), chromatin modifying proteins (SET1 and pp32), and DNA-binding proteins that protect against DNA distortion (HMGB2) [23,27,29,37]. GzmA enters into the nucleus and cleaves SET1, which inhibits NM23-H1 endonuclease activity, causing this complex to nick the DNA, and allowing Trex1 to act as an endonuclease [38]. In the same way, GzmA cleaves and inactivates HGMB2 and Ape1 [26], cleaves the linker histone H1, and removes the tail of core histones, allowing the nucleases to attack [30]. GzmA then cleaves and inactivates Ku70 and PARP-1, both of which are involved in DNA repair through the recognition of single- or double-strand breaks [32,33].

1.2.1.1.2 The Granzyme B Pathway

GzmB is produced by CTL and NK cells, which release it via granules. It binds its receptor, the mannose-6-phosphate/insulin-like growth factor II receptor, and is endocytosed but remains arrested in endocytic vesicles until it is released by PFN. The GzmB pathway relies on caspase activation, unlike the GzmA pathway.

The proteolitic activity of GzmB is similar to caspase activity, cleaving substrates after the aspartate (basic) residues. Caspase 3, 6, 7, 8, 9, and 10 have been found to serve as GzmB substrates in vitro [39–46], but only caspase 3 is believed to be important in vivo [11,12]. As a further mechanism, GzmB can process BID, promoting cyt c release, SMAC/Diablo activation, formation of apoptosis inducing factor (AIF), and release of Omi from the mitochondria. It does this by recruiting the inhibitor of the anti-apoptotic B-cell lymphoma 2 (Bcl-2) family member, especially the Bax protein, to the mitochondrial membrane, leading to apoptosome formation [45–47]. GzmB can also process caspase 3 and 7, initiating the apoptotic process [48]. It has been demonstrated that pro-apoptotic caspase activation happens within minutes of target-cell recognition by CTLs. Unexpectedly, there is a rapid rate of caspase 3/7 biosensor activation following GzmB versus Fas-mediated signal induction in murine CTLs. This Fas-mediated induction is detected after 90–120 minutes in porcine, murine, and human CTLs, consistent with FasL/Fas-induced activity [49]. Recently, key roles for GzmB have been described, positioning it as an allergic inflammatory response of NK [50]. It has also been shown that the major NK cell-activating receptor NKG2D and the NK cell effector are both mediated by GzmB.
1.2.2 Nuclear Features of Apoptosis

The first description of the apoptotic process as a basic biological phenomenon different from necrosis (based not only on morphological criteria) was given by Kerr et al. [51]. The authors described two characteristics of apoptosis: (i) cytoplasmic and nuclear condensation and the disruption of the cell into a number of membrane-bound, well-fragmented pieces; and (ii) formation of apoptotic bodies that are taken up by other cells for degradation. This study shed new light on the apoptotic mechanism as an important process of PCD that regulates several biological processes, including embryogenic development and aging, cell turnover in different tissues, and the control of the immune system. Inappropriate control of apoptosis appears in many human disorders, leading biologists to seek a better understanding of the entire process. Intriguingly, a wide variety of stimuli – both physiological and pathological conditions – can trigger apoptosis. In this section, we address the main biochemical features of apoptosis that focus on nuclear events, including the activation of the execution caspases (i.e., caspase 3, caspase 8, caspase 9, and caspase 10), chromatin condensation, DNA fragmentation, and the formation of apoptotic bodies.

Early evidence described DNA fragmentation as a key feature of apoptosis. Using low concentrations of an exogenous agent like γ-irradiation to induce cell death, it was shown that the DNA of lymphocytes was completely degraded into oligonucleosomal fragments. Further, cells induced with near-physiological concentrations of glucocorticoid hormones showed chromatin condensation as an early structural change. In fact, this particular nuclear morphological change was associated with excision of the nucleosome chains from nuclear chromatin through activation of an intracellular, but non-lysosomal, endonuclease [52]. At this time, it was already known that some members of the caspase family, comprising 12 proteins, are strictly involved in the apoptotic cell death process [53,54]. These are the signals after mitochondrial outer-membrane permeabilization (MOMP) that activate the caspase pathway. However, the interconnection between the nuclear and cytoplasmic events involved in apoptosis became better appreciated when a nuclease protein (Nuc-1), a homolog of mammalian DNAase II, which plays a role in DNA degradation in the nematode Caenorhabditis elegans, was identified as acting downstream of Ced-3 and Ced-4 [56]. In particular, attempts have long been made to understand the link between the executioner caspases and subsequent nuclear apoptotic events, since a variety of death stimuli can activate these proteases, which amplify the signal of cell death.

Caspase 3 is considered the most important protease of the executioner pathway, and can be activated by any of the initiator caspases. Caspase 3 can be activated by caspase 8, which is activated from the death receptor; by caspase 9, which is involved in the mitochondrial apoptotic process; or by caspase 10, which is involved in the PFN/GzmB pathway. Each of these pathways is responsive to a wide range of stimuli capable of amplifying the cellular death signal in an energy-dependent manner. The cleavage of caspase 3 results in the activation of the endonuclease CAD. In apoptotic cells, activated caspase 3 cleaves inhibitor of caspase-activated DNase (ICAD) to dissociate the CAD:ICAD complex, allowing CAD to cleave chromosomal DNA. The CAD:ICAD complex inhibits the CAD activity as DNase. When CAD is cleaved by caspase 3, it can degrade chromosomal DNA like a scissor-like homodimer, cleaving double-strand DNA at nucleosomal linkers [2,3].

CAD is able to condense chromatin and to fragment chromosomal DNA in an irreversible manner that compromises DNA replication and gene transcription, leading
to cell death. Accompanied by chromatin condensation, chromosomal DNA is cleaved into high-molecular-weight fragments of 50–300 kb, which are subsequently processed into low-molecular-weight fragments of approximately 180 bp. Several models have been designed to study the role of CAD in PCD. These studies have shown that the inhibition of CAD activity – for instance, by inducing degradation by a chaperon – can abolish internucleosomal DNA fragmentation. However, the inefficient DNA degradation activity detected in CAD-deficient cells suggests the existence of additional nuclease(s) during apoptosis. An interesting example that links the extrinsic and intrinsic pathways is related to the mammalian endonuclease G (endo G). Endo G is a nuclease that was first identified in the mitochondrial intermembrane space; upon apoptotic stimuli, it may be released from the mitochondria and translocated to the nucleus. The endonuclease activity is responsible for cleaving nucleic acids, representing a caspase-independent apoptotic pathway initiated from mitochondria. In mouse embryonic fibroblast (MEF) cells, taken from a DFF45/ICAD-knockout (KO) mouse, there was no detectable caspase 3-dependent activity, and it was shown that there was minimal DNA fragmentation. Moreover, the induction of apoptosis by ultraviolet irradiation or treatment with cyclohexamide (Chx) led to the release of both endo G and cyt c from the mitochondria to the cytosol and nuclei. The identification of DNA fragmentation has been used as a fundamental biological marker of apoptosis. The main method for detecting apoptotic PCD is known as terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) [57].

There have been several further reports providing evidence for caspase-independent death programming in vitro and in vivo by cathepsins B and D, calpains, and serine proteases. Some of these death routines become evident only when the caspase-dependent pathway is inhibited, particularly in the case of ATP depletion or when using caspase inhibitors.

The evolutionarily conserved execution phase of apoptosis is characterized by cell morphology changes, including cell shrinking, plasma-membrane blebbing, and separation of cell fragments into apoptotic bodies. It is known that the actin–myosin system plays a key role in bleb formation through the activity of the Rho effector protein (ROCK I), which leads to the phosphorylation of myosin light-chain ATPase activity and coupling of actin–myosin filaments to the plasma membranes. Apoptotic bodies consist of cytoplasm-packed organelles that contain nuclear fragment. The integrity of the apoptotic bodies is maintained in order to avoid the release of their cellular constituents into the surrounding interstitial tissue, which would block activation of the inflammatory reaction; this permits the apoptotic bodies to be degraded efficiently within phagolysosomes by macrophages and various surrounding cells.

Although the evolutionarily conserved execution phase of apoptosis has been the theme of many studies, a full understanding of apoptosis at the molecular level is needed if we are to gain deeper insights into its basic and applied biology, particularly regarding new therapeutic strategies.

References


29 Beresford PJ, Kam CM, Powers JC, Lieberman J. Recombinant human granzyme A binds to two putative HLA-associated proteins and cleaves one of them. Proc Natl Acad Sci USA 1997;94(17):9285–90.


