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The regulation of apoptotic cell death

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Abstract

Apoptosis is a fundamental biological phenomenon in which the death of a cell is genetically and biochemically regulated. Different molecules are involved in the regulation of the apoptotic process. Death receptors, coupled to distinct members of the caspases as well as other adapter molecules, are involved in the initiation of the stress signals (*The Indictment*). Members of the Bcl-2 family control at the mitochondrial level the decision between life and death (*The Judgement*). The effector caspases are responsible for all morphological and biochemical changes related to apoptosis including the "eat-me" signals perceived by phagocytes and neighboring cells (*The Execution*). Finally, apoptosis would have little biological significance without the recognition and removal of the dying cells (*The Burial*).

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Phases of the apoptotic death

Apoptosis has been one of the "hottest" topics in cellular and molecular biology in the last few years. Since its morphological definition in the early 70s (1), many of the molecular players have been characterized and the biochemical pathway that regulates this death process has been dissected to the limit. Molecules involved in apoptosis range from proteases, kinases, adapter molecules, transcription factors, and so on. Despite the early findings that apoptosis required *de novo* protein synthesis, we now agree that most of the players are already present in a non-active form just waiting for a sign to initiate a self-destruction program. Cells appear to "sense" that their presence is undesirable to the body as a whole; they silently commit suicide and are swiftly cleared by their neighbors.

This is what apoptosis is about. The morphological characterization that distinguishes apoptosis from necrosis was a pivotal event for the advancement of the field of cell death. However, apoptosis can no longer be viewed as a morphological outcome but rather as an evolutionary conserved physiological program vital for the development and life of multicellular organisms.

The process of apoptosis can be divided into four distinct phases. The first step is what we call the *indictment* and comprises the sensitization of a diverse array of regulatory molecules that will transmit stress signals to the heart of the aerobic living cells - the mitochondria. The second step is defined here as the *judgement* and involves the decision at the mitochondrial level as whether the stress signal is strong enough to justify the condemnation of the cell. The third step is the *execution* of the apoptosis program and

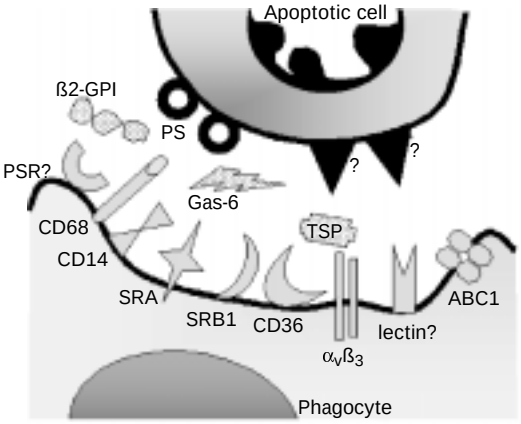

Figure 1 - Molecules involved in the recognition and/or engulfment of apoptotic cells by phagocytes. One of the major features of cells undergoing apoptosis is the externalization of phosphatidylserine (PS) residues. Another putative trait of apoptotic cells is the modification in the glycosylation status of certain membrane proteins. These changes are readily detected by phagocytes either through a direct interaction with specific receptors or via "bridging" molecules such as thrombospondin (TSP), Gas-6 and ß2-glycoprotein I (ß2-GPI). SRA = Class A scavenger receptor; SRB = class B scavenger receptor; ABC1 = ATP binding cassette transporter 1; PSR = as yet unidentified phosphatidylserine receptor.

it is totally dependent on the activation of certain members of the caspases which are responsible for the entire morphological and biochemical outcome of apoptosis. However, apoptosis has very little biological meaning without the fourth and last step in this death track - the recognition and elimination of the apoptotic cells without endangering any other segment of their microenvironment. We call this step the *burial*.

For pedagogic reasons and in order to maintain the sequence of information presented at the XIII Annual Meeting of the Federação de Sociedades de Biologia Experimental we will describe the apoptotic process from the point of cell demise to the initiation of the death signaling cascade.

Recognizing and removing the dead - the biology of apoptosis

The first important concept that we should keep in mind is that apoptosis is not a rare event. On the contrary, the vast majority of the cell deaths that occur during the life-time of an organism involve apoptosis. If that is so, why then is apoptosis rarely seen *in situ*?

Recognition, uptake and degradation of apoptotic cells by their neighbor cells are events that occur very rapidly, making it difficult for us to morphologically detect apoptotic cell death *in situ*. Also important is the fact that this swift elimination somehow prevents the occurrence of an inflammatory process which would be easily noticed both at the macro- and microscopic levels.

Since the dying cells are removed before they lose their cell membrane integrity, it is easy to imagine that they will no longer release their cellular content into the intracellular space and therefore will not initiate any coagulative injury to the adjacent tissue. But this seems to be only part of the reason why the engulfment of apoptotic cells does not elicit an inflammatory reaction. It is very intriguing that whereas the bacterial lipopolysaccharide (LPS) receptor CD14 triggers inflammatory responses upon binding to LPS, the recognition of apoptotic cells via CD14 does not elicit the release of pro-inflammatory cytokines by the same phagocytes (2). So, is it possible that apoptotic cells initiate an anti-inflammatory signaling cascade in phagocytes? The answer to this question was provided recently by two groups showing that recognition of apoptotic neutrophils and peripheral blood lymphocytes by monocyte-derived macrophages induces the release of anti-inflammatory cytokines while blocking both zymosan and LPS induction of pro-inflammatory cytokines (3,4). Consequently, evidence began to emerge suggesting that the failure to induce an inflammatory response during apoptotic death might be due to a great extent to an active mechanism mediated by signaling through phagocyte receptors for apoptotic cells. One of those molecules seems to be CD36, which has been shown to participate in the recognition of apoptotic cells (5-7) and its binding by agonistic antibodies mimics the anti-inflammatory properties of apoptotic cells (3).

Other phagocyte molecules involved in recognition and uptake of apoptotic cells may include class A and class B scavenger receptors, CD68, the $\alpha_v ß_3$ integrin, phagocyte lectins and the ABC1 transporter (8,9) (Figure 1). Some of the phagocyte receptors do not directly interact with molecules present on the apoptotic cell membrane but require the presence of "bridging" molecules.

The best example of this kind of molecule is thrombospondin, which mediates the recognition of apoptotic cells through the $\alpha_v\beta_3$/CD36 complex (7). In addition, β_2-glycoprotein I and gas-6 are two good candidates to perform the "bridging" between apoptotic cells and phagocytes (8). Finally, on the side of the apoptotic cells, the most notable "eat me" signal is the externalization of phosphatidylserine residues normally found on the inner leaflet of the cell membrane (10). This is one of the first events during the apoptosis process (11) and is totally dependent on the activation of the execution program mediated by caspases (12).

Turning on the apoptotic program - a proteolytic finale

Caspases are a special set of cysteine proteases with an unusual specificity for aspartic acid (reviewed in 13,14). The only other protease known to exhibit the same specificity is granzyme B, a serine protease contained in cytotoxic cell granules which functions to initiate the apoptotic death of target cells. All caspases share a similar structure and are synthesized as a precursor with little or no activity which consists of a pro-domain localized at its amino terminus, a large subunit in the middle of the molecule and a carboxy-terminal small subunit (Figure 2). Activation of caspases proceeds by proteolytic cleavage of the constitutively expressed pro-form at two caspase consensus sites, one that clips off the pro-domain and the other that separates the two subunits. The intriguing nature of the cleavage sites indicates that these molecules can undergo autoactivation and also that they can activate each other in an enzymatic cascade similar to the coagulation and the complement cascades. Although the large subunit is the one that contains the catalytic domain, it is only active when associated with the small subunit. In fact, crystallographic studies revealed that the active caspases are tetramers formed by the association of two heterodimers and therefore contain two independent catalytic sites (15-17) (Figure 2).

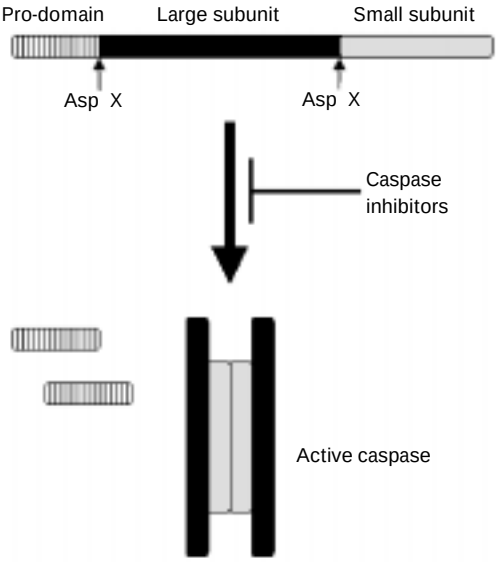

Figure 2 - Diagram of the basic structure of pro-caspases and a model for caspase activation. Caspases are constitutively expressed as a zymogen consisting of a pro-domain localized at its amino terminus, a large subunit in the middle of the molecule and a carboxy-terminal small subunit. These three distinct domains are separated by two caspase consensus sites, one responsible for the cleavage that clips off the pro-domain and the other responsible for the separation of the two subunits. This proteolytic activation is achieved either by auto-processing or by cleavage by another member of the caspases. Active caspases are tetrameric molecules formed by the association of two heterodimers and therefore contain two independent catalytic sites.

How do we know that caspases are the central executioners in the process of apoptosis? First, activation of caspases is a very early event which occurs in all forms of apoptosis. Second, prevention of caspase activation by viral or oncogenic proteins, or peptide inhibitors, blocks all the morphological features associated with apoptosis. Third, administration of recombinant caspases in cell-free systems results in apoptotic cytoplasmic and nuclear changes. However, not all members of the caspases are effector molecules in this program. Caspase-6, -7 and -3 are the ones directly implicated with the execution of apoptotic cells. Caspase-8, -10, -9 and -2 are initiator or regulatory caspases, which means that their activities do not directly account for the morphological features of apoptosis. Instead, the primary role of these caspases is to function as signaling molecules, therefore transducing stress signals capable of activating the effector caspases. Other members include caspase-1, -4, -5, -11, -12, and -13 which appear to be primarily involved in inflammatory processes, probably via pro-

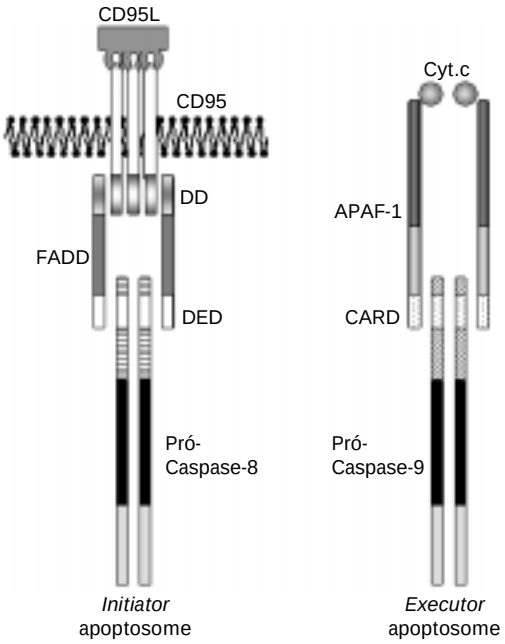

Figure 3 - Proposed structure of the two classes of apoptosomes. Apoptosomes associated with the *indictment* phase of the apoptotic process and therefore related to the death receptors are represented by the CD95L/CD95/FADD/pro-caspase-8 apoptosome. In the case of TNFR1- or DR3-initiated apoptotic cascade, the adapter molecule TRADD links the cytoplasmic domain of the death receptors to FADD/pro-caspase-8 (or -10). The only apoptosome associated with the *execution* phase of apoptosis described so far is the cytochrome c/APAF-1/pro-caspase-9 complex. FADD, Fas-associated death domain; DED, death effector domain; DD, death domains; CARD, caspase recruitment domain; APAF-1, apoptosis activating factor-1; Cyt.c, cytochrome c.

teolytic processing of proinflammatory cytokines such as IL-1 and IL-18 (18-20).

During apoptosis stimulation, caspases are first activated in the context of multimolecular structures called apoptosomes, which can be divided into two subgroups (Figure 3). The first one is related to cell death initiated by cross-linking of so-called "death receptors" such as CD95 and TNFR1 (see below) while the second is involved in genotoxic stimulation and is composed of three different molecules, i.e., apoptosis activating factor-1 (APAF-1), cytochrome c and pro-caspase-9. In normal cells, inactive forms of APAF-1 and caspase-9 are present in the cytoplasm whereas cytochrome c participates in the electron transport chain at the mitochondrial level. Upon stress signals, cytochrome c is released from the mitochondrial intermembrane space into the cytoplasm where it binds to APAF-1. This interaction changes the conformation of APAF-1 which subsequently associates with procaspase-9, thereby inducing its activation, the activation of the effector caspases (in particular caspase-3) and the initiation of the proteolytic cascade observed in apoptosis (21).

The fact that a proteolytic activity inside the cell is responsible for its demise led us to think that the main role of caspases was to destroy intracellular proteins in such a way that the cell would not be able to function any longer (22). This view is only partially true. Substrates such as DNA-PK_{cs}, U1snRNP-70k, lamins, fodrin and the focal adhesion kinase are indeed neutralized by caspase-mediated proteolytic cleavage, thereby contributing to inactivation of both the DNA repair and the RNA splicing machinery, disassembly of the nuclear lamina and alteration in cytoskeleton structure, and cell-cell contact. However, some of the substrates are instead activated by caspase cleavage. For instance, cleavage of the p21-activated kinase PAK2 generates a constitutively active fragment that mediates membrane blebbing and the formation of apoptotic bodies (23). Similar activation was also reported for gelsolin (24). Other examples include the cleavage of the Bcl-2 family member Bid (25) and one of the subunits of the DNA fragmentation factor, iCAD/DFF45, so that the other subunit (CAD/DFF40) is released resulting in oligonucleosomal DNA fragmentation (26).

Judgement in the intracellular realm - the mitochondria decide if cells live or die

So, does this mean that if we block the activity of caspases we will prevent every sign of apoptosis and therefore keep the cells alive? The answer is yes in two situations: a) when apoptosis induction involves an initiator caspase, as is the case for apoptosis triggered by cross-linking of "death-receptors" (see below), and b) during developmental cell death when it appears that up-regulation of the expression of effector caspases, such as caspase-3, may lead to the oligomerization of these molecules thereby activating them and turning on the apoptosis program.

But the answer is no when apoptosis occurs as a consequence of genotoxic stimulation. In this case, pan-caspase inhibitors do block the apoptosis phenotype but are unable to prevent the cell demise which occurs by a non-apoptotic mechanism, apparently coordinated by the mitochondria (27-30).

The experimental results obtained in the last few years were fundamental to define mitochondria as stress sensors and as the rheostat that determine whether the cells live or die. In our opinion, perhaps the most important cell death event generated at the mitochondrial level is the release of cytochrome c from the mitochondrial intermembrane space to the cytosol. This brings about at least three major deleterious pathways for the cells. First, as we mentioned above, cytochrome c will associate with APAF-1 and initiate the activation of the effector caspases leading to apoptosis. Second, cytochrome c release from mitochondria disrupts the electron transport chain with consequent impairment of the production of energy. If cells are not able to compensate for such disturbance, they will certainly die regardless of the activation or not of the caspase cascade. There will be just not enough energy to sustain the basic metabolic reactions that keep cells alive. Third, this same event will increase the generation of reactive oxygen species which are potent cytotoxic agents. Thus, these observations strongly suggest that it is highly unlikely that a cell will remain viable and continue to proliferate when the stress is enough to signal the mitochondria to release their cytochrome c. Indeed, we and others have found that such stress signals as cytotoxic drugs, UV- or γ-irradiation, enforced expression of Bax or c-myc, glucocorticoids, etc., lead to cytochrome c release and cell death despite the complete inactivation of the apoptosis program by pan-caspase inhibitors (27-30). Therefore, we would like to consider the translocation of cytochrome c from mitochondria to the cytosol as the moment when a cell is committed to death. It is important to point out, however, that other mitochondrial events such as the release of other pro-apoptotic factors or the opening of the so-called mitochondrial permeability transition pore may also contribute to this *commitment point* (discussed in 31,32). In this regard, it still remains elusive how cytochrome c escapes from the mitochondria.

Like the effector caspase-mediated apoptotic program, the mitochondria-derived commitment decision is also molecularly regulated. Pro- and anti-apoptotic members of the Bcl-2 family control the mitochondrial rheostat of cell death (33). Recent evidence pointed at two possible survival mechanisms in the mammalian system that operate at the mitochondrial level. One resembles the CED-9/CED-4/CED-3 pathway described in *Caenorhabiditis elegans*, and proposes that Bcl-x_L binds APAF-1 thus preventing the activation of caspase-9 and subsequent apoptosis (34). The other implicates anti-apoptotic members such as Bcl-2 and Bcl-x_L in the maintenance of mitochondrial integrity (reviewed in 35) and the regulation of their membrane permeability to small pro-apoptotic molecules such as cytochrome c (36,37) and apoptosis inducing factor (AIF) (38). It is not completely understood how Bcl-2 members control mitochondrial integrity but, based on the structure of Bcl-x_L which resembles pore-forming bacterial toxins and the fact that Bcl-2, Bcl-x_L and Bax do form ion channels in lipid bilayers, it is conceivable that the control of small conductance ion channels by these molecules may contribute to the preservation of mitochondrial physiology (discussed in 32,39). Based on these observations, it is not surprising that enforced expression of Bax, a pro-apoptotic member of this family and a channel-forming protein, directly induces destructive changes in mitochondria which result in cell death not only in mammalian cells but also in yeast, where death occurs in the absence of any caspase-like activity. In fact, Bax-in-

duced cell death is counteracted by Bcl-2 or Bcl-x_L but not by inhibitors of caspases (30,40). There is, of course, another way the pro-apoptotic members of Bcl-2 family can "sensitize" a cell for death. They can cause the release of cytochrome c.

In any case, it is important to note that whereas the effector caspases are the central executioners in the apoptosis process, mitochondria are the arbiter of the life and death decision inside a cell. Also, in many instances apoptosis would be more properly considered as a disposal program rather than a killer agenda.

Indictment by the environment - signaling through death receptors

We still know very little about the nature of the pro-death signals that have an impact on the mitochondria. However, as we previously mentioned in this review, initiator caspases are able to transduce signals that stimulate pro-apoptotic changes in the mitochondria. One of the initiator caspase-dependent signals involves the proteolytic activation of Bid, a pro-apoptotic member of the Bcl-2 family, by caspase-8. Activated Bid acts on the mitochondria to orchestrate the release of cytochrome c and other cell death events which culminate in apoptosis (25,41).

In comparison to pro-caspase-9 which is activated in the context of the cytochrome c/APAF-1 apoptosome, caspases-8, -10 and -2 are activated in apoptosomes formed by some members of the tumor necrosis factor receptor (TNFR) family, known as "death receptors", and adapter molecules such as FADD (Fas-associated death domain), TRADD (TNFR-associated death domain), RIP (receptor-interacting protein) and RAIDD (RIP-associated Ich-1/CED-3 homologous protein with death domain). Five death receptors have been described so far in mammals - CD95 (Fas/Apo-1), TNFR1, DR3 (Apo-3/Wsl/TRAMP/LARD), DR4 and DR5 (Apo-2R/TRAIL-R2/TRICK-2/KILLER), all of them presenting typical extracellular cysteine-rich domains that characterize the TNFR members and additional intracellular "death domains" (DD) (reviewed in 42). Interestingly, every ligand for such molecules belongs to the TNF superfamily and is produced as a trimer. Consequently, these death signals are initiated by trimerization of the receptors, resulting in the association of their DD. This association leads to homotypic interaction with adapter proteins containing DD. FADD binds to CD95 whereas TRADD binds to TNFR1. Besides the DD, FADD has a different domain called DED ("death effector domain") which specifically interacts with DED present in the prodomain of caspases-8 and -10. The apoptosome CD95/FADD agglutinates caspase molecules, thereby stimulating their proteolytic activity and initiating the apoptosis cascade (43). TRADD does not contain DED and therefore cannot bind to caspases. Thus, in order to transduce a death signal, TNFR1/TRADD needs to bring FADD to the complex forming the TNFR1/TRADD/FADD apoptosome which afterwards will recruit caspase-8 or -10 (44). In addition, TNFR1 may activate a FADD-independent death pathway by engaging a different TRADD-binding adapter protein called RIP (45). RIP associates further with RAIDD (46) which has yet another domain responsible for homotypic adhesion. This domain, called CARD (caspase recruitment domain) (47), is also present in APAF-1 and in caspases-9 and -2 (48). Whereas the activation of caspase-8 and -10 is dependent on FADD, caspase-2 is activated in a FADD-independent manner, upon formation of the TNFR1/TRADD/RIP/RAIDD apoptosome. However, it is important to note that the predominant death pathway in both cases is the FADD/caspase-8.

The death signals initiated by the interaction of Apo3L with DR3 seem to be similar to those triggered by TNFR1. In comparison, apoptosis initiated by Apo2L/TRAIL-induced crosslinking of either DR4 or DR5

seems to be mostly independent of FADD since cells from FADD-deficient mice are fully sensitive to DR4-induced apoptosis, despite the fact that they display a strong resistance to CD95-, TNFR1- and DR3-induced cell death (49).

To further complicate the puzzle of molecular interactions involved in death receptor-mediated apoptosis, there is another set of membrane proteins designated decoy receptors (DcR). These molecules, represented by DcR1 (TRID/TRAIL-R3/LIT) (50) and DcR2 (TRAIL-R4/TRUNDD) (51), resemble DR4 and DR5 in their extracellular domain but lack a proper intracytoplasmic tail. Therefore, they compete for Apo2L/TRAIL ligation without transducing any kind of biochemical signal.

Since initiator caspases are directly linked to the death receptor apoptosomes and are required for the proper propagation of the death signal, it is not surprising that pan-caspase inhibitors not only block CD95 or TNFR1-induced apoptosis but also confer resistance to cell death in these circumstances by preventing the mitochondrial event we described as the commitment point.

At least two observations indicate that, even if caspase-8 can bypass the need for mitochondrial events and induce apoptosis by directly activating the effector caspases such as caspase-3, the death receptor-associated caspases seem to induce apoptosis through a mitochondria-dependent mechanism in most cases. First, in many, but not all cells, CD95-induced apoptosis is blocked by Bcl-2, and this apoptotic pathway is even better prevented by Bcl-x_L (52,53). Second, the addition of mitochondria to cell-free systems results in a full-blown nuclear apoptosis in conditions where low doses of caspase-8 are not sufficient to induce the typical nuclear changes. In this system, Bcl-2 is able to block apoptosis induced by low but not high doses of caspase-8 (54).

Concluding remarks

Cells may die either by apoptosis or by non-apoptotic mechanisms. If damage is too violent (pathologic), cells have no choice but to undergo the genetically uncontrolled necrotic form of death - they lose membrane integrity, swell and may quickly burst, releasing their cellular contents. On the other hand, when damage is more subtle (physiologic) it will generate biochemical signals that converge on the mitochondria, special organelles that act as "stress sensors" having the onus to decide whether the cell may continue to live or should die. In this case, death normally occurs through apoptosis. However, it is important to note that whenever a cell moves forward to the mitochondrial *commitment point* it can no longer be rescued. Not even the complete blockage of the activity of the effector caspases will rescue this cell from its demise - instead of undergoing the more common apoptosis, the cell will die by a non-apoptotic mechanism. To sum up, the decision between life and death is up to the mitochondria and is regulated by members of the Bcl-2 family. In contrast, the execution of the apoptotic program and the consequent emergence of all morphological changes peculiar to apoptosis are dependent on the effector caspases.

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