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Apoptotic Pathways: The Roads to Ruin

Minireview

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Introduction

Nearly all physiological cell deaths in animals proceed by the process of apoptosis, during which the dying cells vanish without a trace, silently cleared without any accompanying inflammatory response. Like the last scene of a noir mystery, we can now line up the various molecular suspects in this cell death. Here, we will examine recent knockouts and other studies that shed light on the pathways that lead to the death of a cell by apoptosis.

The final stage of apoptosis, called execution, occurs through the activation and function of caspases, a highly conserved family of cysteine proteases with specificity for aspartic acid residues in their substrates. It is the cleavage of certain key substrates that orchestrates the death and packaging of the cell for clearance. The keys to understanding the molecular basis of apoptosis lie not only in the identification of these critical caspase substrates, but also in learning how the caspases that cleave them come to be activated.

Caspase Activation: The Procaspase Always Cuts Twice

Caspases are constitutively present in most cells, residing in the cytosol as a single chain proenzyme. These are activated to fully functional proteases by a first proteolytic cleavage to divide the chain into large and small caspase subunits and a second cleavage to remove the N-terminal domain (prodomain). The subunits assemble into a tetramer with two active sites (see Figure 1).

Since proteolytic cleavage generates the mature caspases, one way in which these enzymes are activated is via the action of proteases, including other caspases. Thus, caspases can function in an activation cascade. Alternatively, caspases can be activated by other proteases such as granzyme B, which is introduced into cells by cytotoxic lymphocytes and triggers apoptosis by cleavage and activation of caspases such as caspase-3. Caspases with short prodomains lacking protein interaction motifs (caspases-3, -6, -7) are probably activated predominantly through the action of other proteases. These are sometimes referred to as "downstream," "effector," or "executioner" caspases.

While caspase–caspase interactions are almost certainly of central importance, the initial activation of caspases during the apoptotic process must involve something more. The key is that the procaspases possess activity, albeit less than that of the active caspases; e.g., procaspase-8 has approximately 1%–2% of the activity of the mature caspase-8 enzyme. When procaspase-8 molecules are aggregated, this is sufficient to allow auto- or transprocessing to produce caspase-8 (Muzio et al., 1998; Yang et al., 1998). Thus, procaspase aggregation is an important step leading to caspase activation, which in turn is amplified by cleavage and activation of downstream caspases.

Procaspase aggregation is mediated by the binding of adaptor molecules to protein interaction domains in the prodomains of some caspases. Two general types of interaction domains have been identified. Procaspases-8 and -10 each contain two tandem death effector domains (DEDs), while procaspases-1, -2, -4, -5, and -9 contain caspase recruitment domains (CARDs). In each case, the procaspases bind to adaptor molecules containing similar domains, and these either directly aggregate or interact with other molecules. Although they are unrelated, CARDs and DEDs have similar structures, composed of six closely packed, amphipathic antiparallel α helices, a structure that is also found in the death domains of Fas and the p75 NGF receptor (Chou et al., 1998; Eberstadt et al., 1998).

The result in each case of caspase recruitment is the formation of a complex called an "apoptosome" that functions to mediate the activation of the caspase (Figure 1). Two distinctly different processes have been delineated.

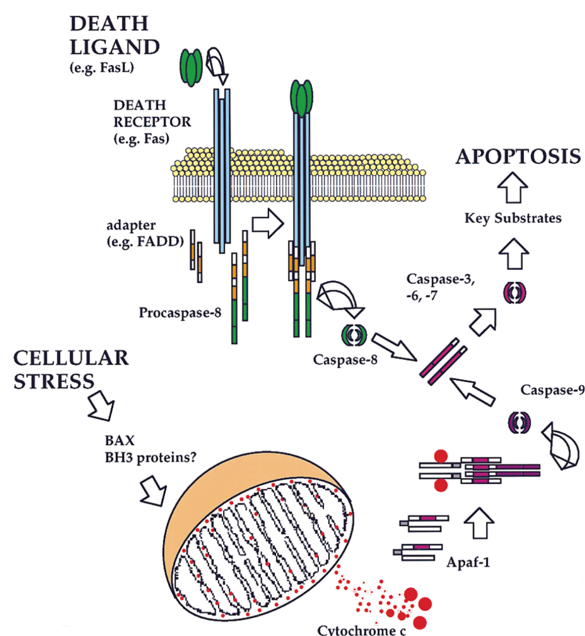

Figure 1. Two Pathways of Caspase Activation and Apoptosis

Two independent apoptosis pathways are presented that converge on the activation of "downstream" caspases (-3, -6, -7), key substrate cleavage, and apoptotic death. The first involves ligation of death receptors by their ligands, resulting in the recruitment of adaptor proteins and procaspase molecules. The complex is an "apoptosome" in which the aggregated procaspase transactivates. The active caspase (e.g., caspase-8) then acts to cleave and activate the downstream caspases. In the second pathway, various forms of cellular stress trigger mitochondrial release of cytochrome c, which binds to Apaf1, which in turn self-associates and binds procaspase-9, resulting in an apoptosome. Transactivation of the complexed procaspase-9 to active caspase-9 follows, and the caspase then cleaves and activates downstream caspases. In this model, there is no cross-talk between the pathways, and ligation of death receptors does not affect mitochondrial function in a relevant manner.

Spellbound: Mechanisms of Caspase Aggregation

Upon their ligation, death receptors such as Fas and the TNF receptor-1 (TNFR1) recruit and oligomerize adaptor proteins and procaspases. FADD, which contains a DED, is recruited directly to ligated Fas (and indirectly to ligated TNFR1), resulting in the recruitment and autoactivation of caspase-8. Similarly, the adaptor protein RAIDD (which has a CARD domain) associates with TNFR1 and thus may promote aggregation and activation of procaspase-2. Another adaptor, CARDIAK/RIP2, binds via CARD–CARD interactions to procaspase-1, possibly leading to activation.

A related but different way that adaptor proteins function to activate caspases is seen in the case of Apaf1, a mammalian homolog of the *C. elegans* protein CED-4. In the nematode, it appears that CED-4 promotes developmental cell death by binding to the procaspase CED-3. CED-3 autoactivation then occurs, probably via aggregation or ATP-driven conformational changes in CED-4. Similarly, Apaf1 can bind the prodomain of procaspase-9 (via a CARD–CARD interaction) while a different region of Apaf1 self-associates, resulting in procaspase aggregation and activation (Srinivasula et al., 1998). However, the binding of Apaf1 to procaspase-9 and to itself only occurs in the presence of cytochrome c. Apaf1 also binds ATP and dATP, and one or the other are required for efficient cytochrome c–induced caspase-9 activation. Removal of the C-terminal WD repeat domain of Apaf1 eliminates the requirement for cytochrome c, leading to the idea that cytochrome c activates Apaf1 function by binding to this region (Srinivasula et al., 1998), although this has not been formally demonstrated. The truncated Apaf1 activates caspase-9 but apparently does not release it, raising the possibility that conformational changes (perhaps ATP dependent?) may play additional roles in the function of this apoptosome. This cytochrome c/Apaf1 pathway and the death receptor pathway discussed above both converge on the activation of the downstream caspases (see Figure 1).

While procaspase aggregation and transactivation appear similar in both cases (death receptors versus Apaf1), the involvement of cytochrome c introduces a fundamental difference. Only cytochrome c that has been assembled in the mitochondria (i.e., with the attached heme) functions to activate Apaf1 (Yang et al., 1997), and thus mitochondrial release of cytochrome c appears to be critically involved in Apaf1 function.

What triggers the release of cytochrome c? In most cases the release of cytochrome c proceeds independently of caspase function (Bossy-Wetzel et al., 1998). The effect may or may not be associated with a drop in inner mitochondrial membrane potential corresponding to the opening of the inner membrane permeability transition (PT) pore complex (Green and Reed, 1998). The proapoptotic Bcl2 family member Bax can directly cause mitochondria to release cytochrome c (Jurgensmeier et al., 1998). Bax shuttles from its cytoplasmic location to the mitochondria upon induction of apoptosis (Wolter et al., 1997). The ability of some Bcl2 family proteins, including Bax, to form ion channels has fostered the idea that these proteins open pores or produce breaks in the outer mitochondrial membranes, allowing exit of cytochrome c.

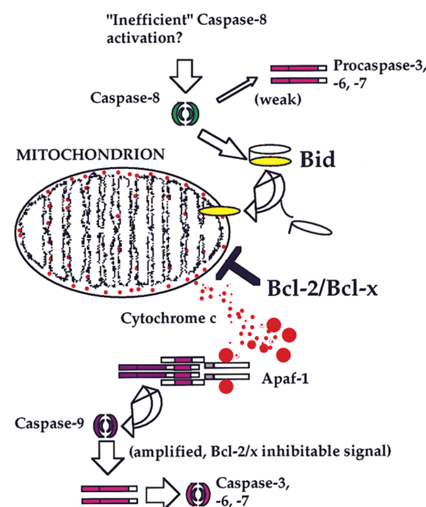

Figure 2. Cross-Talk between the Apoptotic Pathways

Active caspase-8 (e.g., by ligation of death receptors) cleaves Bid, producing a C-terminal fragment that then binds to mitochondria and induces release of cytochrome c. The cytochrome c then functions to activate Apaf1 and procaspase-9 processing, which in turn cleaves and processes downstream caspases. It is hypothesized that inefficient activation of caspase-8 favors this pathway over direct caspase-8-mediated activation of downstream caspases. Bcl2 and BclX act to prevent cytochrome c release and thus interfere with this pathway.

Pathway Cross-Talk: Sorry, Wrong Number

The two basic pathways depicted in Figure 1 make predictions about how apoptosis is regulated under different circumstances. Anti-apoptotic Bcl2 family proteins (e.g., Bcl2, $BclX_L$) bind to mitochondria and inhibit the release of cytochrome c (Kluck et al., 1997; Yang et al., 1997). Therefore, apoptotic signaling via the death receptor pathway should be resistant to Bcl2. However, it seems that Bcl2 and BclX can interfere with Fas- and TNFR-mediated apoptosis in cells in which Fas/FADD/procaspase-8 recruitment is inefficient (Scaffidi et al., 1998). Since Fas and TNFR ligation is associated with release of cytochrome c, it seems likely that cytochrome c release is important in those cases where Bcl2 interferes with death. This raises the possibility of cross-talk between the pathways, such that caspase-8 activates caspase-9 via the mitochondria.

Recently, a mechanism for such cross-talk was provided. Bid, a proapoptotic member of the Bcl2 family, is directly cleaved by caspase-8 and the C-terminal fragment acts on mitochondria to trigger cytochrome c release (Li et al., 1998; Luo et al., 1998) (Figure 2). Depletion of Bid from cytosolic extracts disrupts the ability of caspase-8 to trigger cytochrome c release in vitro. But is this an important mechanism whereby caspase-8 activates downstream caspases? In *Xenopus* cytosolic extracts, the ability of small amounts of caspase-8 to trigger activation of a caspase-3-like activity was dependent on the presence of mitochondria (Kuwana et al., 1998). This suggests the possibility that caspase-8 cleaves and activates Bid more efficiently than procaspase-3, and in doing so enlists the cytochrome c/Apaf1

pathway to amplify the caspase-8 function. In the absence of mitochondria, caspase-8 processes procaspase-3 with identical kinetics whether or not cytosol is present (Stennicke et al., 1998), and thus other caspases do not effectively serve as intermediates or amplifiers of caspase-8 activity. Presumably it is only in those cells where the mitochondrial amplification loop is important that antiapoptotic Bcl2 family members can suppress Fas/TNFR-induced apoptosis (see Figure 2).

Double Indemnity: Redundancy in Apoptotic Pathways

How generally important are these apoptotic pathways? Keeping in mind the possible problems of compensation and redundancy, knockout studies are beginning to give us a new respect for the executioner and its components. The effect of targeted disruption of Apaf1 is perhaps the most dramatic (Cecconi et al., 1998, and Yoshida et al., 1998 [both in this issue of *Cell*]). In these mice, defects are found in essentially all tissues whose development depends on cell death, including loss of the interdigital webs, formation of the palate, control of neural cell number, and development of the lens and retina. Unless additional mechanisms for Apaf1 activation exist that have not been identified, these results suggest that these developmental cell deaths are also dependent on mitochondrial release of cytochrome c.

Interestingly, however, some forms of apoptosis were partially or completely intact in these mice. Fas-mediated apoptosis is fully functional in T cells (Yoshida et al., 1998) although it may be partially lost in embryonic fibroblasts (Cecconi et al., 1998), consistent with the possible role of Bid/mitochondrial signaling through Apaf1 in the latter but not the former. Cell death induced by glucocorticoids, staurosporine, and other agents was still partially intact in the absence of Apaf1, and where examined this appeared to be apoptosis.

It is formally possible that this remaining Apaf1-independent apoptosis is via death receptor signaling, since a number of recent studies have shown that cellular stress can induce expression of death ligands (e.g., Fas-ligand) and subsequent apoptosis (Kasibhatla et al., 1998). However, it seems more likely that other undiscovered Apaf1-like molecules maintain apoptotic responses in these cases.

Caspase knockouts are also extremely interesting, although here the picture is made much more complicated by the number of different caspases. Little or no effect on apoptosis was observed upon targeted disruption of caspase-1, -2 (Bergeron et al., 1998), or -11 (Wang et al., 1998) although knockouts of caspase-1 and -11 did have profound effects on cytokine (e.g., IL-1) processing. Of course this does not rule out roles for these caspases in some forms of apoptosis, since others may perform redundant functions.

In contrast, the knockouts of caspase-3 and -9 (Hakem et al., 1998; Kuida et al., 1998) had profound developmental effects, especially in the brain. Cells from these animals displayed resistance to a number of forms of apoptosis as well. The relative importance of these two caspases was elegantly examined in different cells under different conditions (Hakem et al., 1998), and the results illustrate the complexities of these interactions, in that one or both of these caspases may be essential in a given setting but not in others.

It is important, however, that these caspase knockouts did not accurately mimic the Apaf1 knockouts. This indicates that either Apaf1 can act via a caspase other than procaspase-9, or that it plays additional roles beyond that of execution. The latter is unlikely, however, since all of the embryonic defects examined corresponded to a lack of apoptosis (Cecconi et al., 1998; Yoshida et al., 1998). The idea that another caspase may substitute for procaspase-9 in many cells is appealing, but currently none of the other caspases appear to be activated by Apaf1 (Srinivasula et al., 1998). We might predict, though, that the prodomain of this replacement caspase will contain a CARD domain. Alternatively, it is possible that CARD-containing adaptor proteins bridge Apaf1 to other procaspases. In any case, there are additional apoptosomes waiting to be uncovered.

Dial "M" for Mitochondria: Why Aren't These Cells Dead?

One should expect that a knockout of a gene that encodes one of the central elements of the executioner should manifest as cell survival. But a problem emerges when one considers that triggers of the apoptotic process presumably target mitochondria for cytochrome c release, prior to any involvement of Apaf1, caspase-9, or caspase-3 (Figure 1). The result is the disruption of electron transport, generation of reactive oxygen species, and release of cytochrome c to trigger apoptotic events. In the absence of Apaf1, caspase-9, or caspase-3, why doesn't the cell die in a caspase-independent fashion, due to the disruption of mitochondrial function?

First, it is not certain that this isn't the case. Although the cells of the interdigital webs do not die on schedule in the Apaf1 knockouts, they do apparently die one or two days later (Cecconi et al., 1998). Similarly, the resistance to cell death in *Apaf1*$^{-/-}$ cells was only examined in a short (24 hr) time frame; subsequent caspase-independent death might take longer. However, although alternative Apaf1-independent apoptotic pathways might be engaged later, it does not appear that cells are committed to die by the apoptosis-related mitochondrial changes that precede the involvement of Apaf1.

Experimental evidence supports the idea that cell death can proceed in the absence of caspases. While caspase inhibitors block the apoptotic phenotype, death in cell lines nevertheless proceeds when induced by a variety of agents or conditions (Green and Reed, 1998). Thus, the cells are committed to die even before caspases become active. An exception is cell death induced by ligation of death receptors; in this case the commitment is dependent on caspases, and inhibitors therefore maintain cell viability.

The mitochondrial PT that accompanies apoptosis in many cases is a major candidate mechanism for caspase-independent death. While PT can occur in the absence of active caspases, in many cases it appears to be caspase dependent, even when cytochrome c release is not (Bossy-Wetzel et al., 1998). Activated Bid was found to be capable of inducing cytochrome c release from mitochondria without a loss of mitochondrial transmembrane potential (Luo et al., 1998). Similarly, procaspase-9$^{-/-}$ cells could be stimulated to release cytochrome c without a detectable PT (Hakem et al., 1998). The possible role of a reversible PT is not excluded.

Thus, while cycling cells (including transformed cells) cannot survive the caspase-independent mitochondrial changes that contribute to apoptotic pathways, possibly other cells in the body can. Either mitochondrial function remains intact following the cytochrome c release, or new mitochondria are generated de novo in the surviving cells. Apparent mitochondrial biogenesis in cells triggered to undergo apoptosis has been documented (Mancini et al., 1997).

If, however, this recovery does not readily occur in dividing cells, then this could explain why defects in the apoptotic executioner are not frequently found in cancers. In such cells the lack of caspase activation might not give a significant survival advantage, if the mitochondrial events would prove lethal anyway. Thus, tumor cells can be induced to undergo apoptosis and maintain the apoptotic machinery intact, because the loss of this machinery is not selected. In contrast, tumors do express antiapoptotic proteins that act at the level of the mitochondria (e.g., Bcl2, BclX), since these maintain viability as well as inhibiting apoptosis. Of course, these ideas must await a more thorough examination of oncogenesis in Apaf1- or caspase-deficient cells.

Conclusions

During development, upon cellular stress, or in response to disruption of cell cycle controls, cells engage the pathways that activate endogenous caspases which orchestrate the cells' demise so that they will be effectively and silently cleared. While the nature of these pathways is now coming into view, we still have little idea of how developmental signals or stress responses engage these pathways, or how transcription contributes to them in many cases. There is more to learn about the triggers, the pathways, and the central mechanisms, and just how far down the road to ruin a cell can travel, and still turn back.

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