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Apoptotic Pathways: Ten Minutes to Dead

Minireview

Douglas R. Green*
La Jolla Institute for Allergy and Immunology
10355 Science Center Drive
San Diego, California 92121

For more than a decade, it has been apparent that apoptosis and other forms of cell death are often controlled at one or more crucial steps involving the mitochondria. Recent findings, including an elegant investigation in a recent issue of *Cell* (Hao et al., 2005), have helped to elucidate fundamental aspects of this involvement while raising puzzling new questions about mitochondrial routes to cellular demise. The emerging, if preliminary, perspective these new studies provide may represent either a refinement of our views of how cells die or, perhaps, the beginnings of what amounts to a reformulation of our ideas.

The basic mitochondrial pathway of apoptosis in vertebrates (Danial and Korsmeyer, 2004) begins with the permeabilization of the mitochondrial outer membrane by proapoptotic members of the Bcl-2 family, resulting in a release of proteins from the intermembrane space into the cytosol. Among these released proteins is cytochrome c, which interacts with monomeric APAF-1 to facilitate a conformational change in the latter, leading to its oligomerization and recruitment of caspase-9 to form the apoptosome (Figure 1). The associated caspase-9 is thereby activated, and this in turn cleaves and activates the executioner caspases-3 and -7. These then cleave key substrates in the cell to produce the cellular and biochemical events we see as apoptosis. Other released proteins facilitate caspase activation through inactivation of endogenous inhibitors of caspases, the inhibitor of apoptosis proteins (IAPs). While activated executioner caspases clearly kill cells via apoptosis, inhibition of these proteinases only transiently protects cells; once the mitochondria permeabilize, death will proceed regardless of caspase activation, either due to other toxic mediators released from the mitochondria or eventual loss of essential mitochondrial functions (Chipuk and Green, 2005).

The entire process, from the initial trigger to the destruction of the cell, can take hours or even days. But the events that concern us here, beginning with the first mitochondrial changes and culminating in the activation of caspases, often take about ten minutes, and it is strongly suspected that once these events proceed to the point of executioner caspase activation without constraint, the death of the cell may be inevitable. Here, I discuss recent findings regarding these ten minutes, including caspase activation and the signals that cause mitochondrial outer membrane permeabilization (MOMP). Along the way we explore what may be simple detours from the road to ruin, or might represent important alternate routes to cellular destruction.

*Correspondence: doug@liai.org

Shadow of a Doubt: Cytochrome c and APAF-1

At the core of the mitochondrial pathway of apoptosis is the cytosolic function of cytochrome c to activate caspases via APAF-1, a function that is distinct from that of mitochondrial cytochrome c in electron transport. This distinction allowed Hao et al. (2005) to generate a mouse expressing a mutant cytochrome c (K72A) capable of sustaining mitochondrial respiration but lacking APAF-1-activating potential. Developmental abnormalities in these mice generally resemble those of APAF-1 and caspase-9-deficient animals, providing support for the core mitochondrial pathway in an especially elegant manner.

Presently, we know of only one way in which caspase-9 can be activated, leading to apoptosis: cytochrome c induces the oligomerization of APAF-1, which recruits and dimerizes caspase-9, and this association activates the caspases. It is important to note that unlike the executioner caspases, initiator caspases such as caspase-9 cannot be activated by being cleaved—activation requires active dimerization by an adaptor molecule (Fuentes-Prior and Salvesen, 2004). Therefore, while cleavage of caspase-9 can be observed in a variety of settings, the only known adaptor/activator for caspase-9 is APAF-1 that has been triggered by cytochrome c.

By careful examination of the phenotypes and properties of mouse mutants of the three key players (cytochrome c, APAF-1, caspase-9), however, we may get glimpses of alternative routes to APAF-1 and/or caspase-9 activation. Depending on the strain background, these animals can often survive to birth and mature, albeit with growth and behavioral abnormalities that are due to developmental defects in the hypothalamus (Hao et al., 2005)). An analysis of the response to different apoptosis inducers in cytochrome c mutant and APAF-1 null thymocytes showed intriguing differences that may suggest alternatives to the canonical pathway. While cell death in APAF-1 null thymocytes was delayed in response to multiple proapoptotic agents, the cytochrome c mutant cells died under some of these conditions (Hao et al., 2005). Can APAF-1 be activated independently of cytochrome c?

If so (and I consider the alternative below), it may be that such a mechanism constitutively activates APAF-1 at low levels. A recent structural and biochemical study of truncated APAF-1, lacking its WD domain, shows the role of dATP (and ATP) in APAF-1 activation and how this may relate to cytochrome c binding (Riedl et al., 2005). The nucleotide binding site in APAF-1 is essential for its function, and hydrolysis of dATP (and ATP) by the molecule results in its unfolding. The APAF-1 WD presumably masks the nucleotide binding site, and cytochrome c remains the only molecule known to react and unmask it. However, if the nucleotide can somehow gain access to the site independently of cytochrome c, APAF-1 activation should be possible. One way might be the expression of an isoform of APAF-1 lacking this inhibitory function of the WD region; however, none of the several isoforms of APAF-1 that have been described have such constitutive activity.

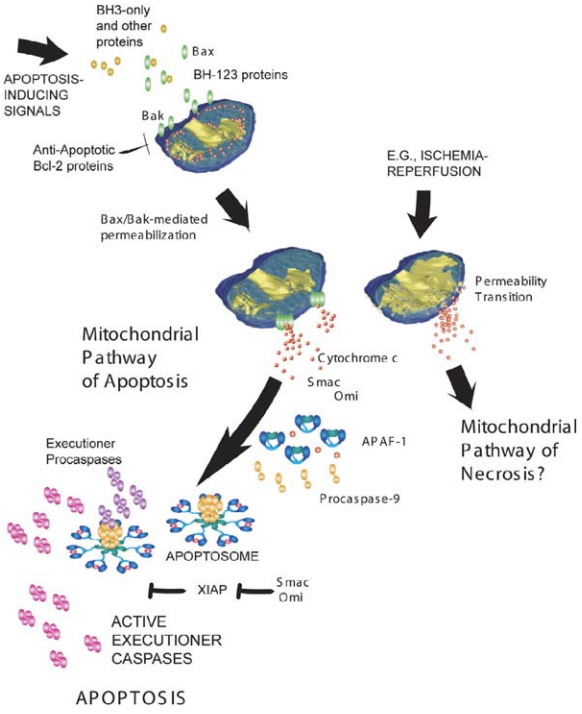

Figure 1. Mitochondrial Roads to Ruin

Two related ways in which mitochondria contribute to cell death are pictured. In the first, mitochondrial outer membrane permeabilization (MOMP) occurs when the proapoptotic Bcl-2 family proteins Bax and/or Bak are activated by BH3-only proteins in response to apoptosis-inducing signals. This results in the release of proteins of the mitochodrial intermembrane space, including cytochrome c, Smac/DIABLO, and Omi/HtrA2. Cytochrome c activates APAF-1, which oligomerizes to form an apoptosome, that in turn recruits and activates caspase-9. The activated caspase-9 cleaves and activates executioner caspases. Inhibitor of apoptosis proteins (IAPs) block caspase-9 function, and this is disinhibited by Smac and Omi. A second pathway to cell death is triggered by conditions that engage the mitochondrial permeability transition in the mitochondrial inner membrane, leading to matrix swelling and rupture of the outer membrane. This death pathway appears to engage a necrotic form of death. While evidence suggests that these two mitochondrial pathways are distinct, it is suspected that there is significant overlap between them.

Nevertheless, if such low level activation of APAF-1 does indeed occur, the cytochrome c-independent activation of caspase-9 in this setting might be expected to be regulated by the IAPs, such as X-linked IAP (XIAP), that bind and block caspase function. The situation may be analogous to what is thought to occur in *Drosophila* apoptosis; a constitutively active APAF-1 homolog, ARK, triggers the caspase-9 homolog, DRONC, whose activity is held in check by an IAP (DIAP-1) (Danial and Korsmeyer, 2004). Apoptosis proceeds only when the caspase-IAP interaction is blocked. In the case of vertebrate cells, several proteins are known that can compete for IAPs and thereby derepress caspase activation. These proteins, such as Smac/DIABLO and Omi/HtrA2, are all found sequestered in the mitochondrial intermembrane space, and they are released upon MOMP. Therefore, in our speculative scenario, apoptosis might proceed via engagement of the mitochondrial pathway, independently of cytochrome c.

This scenario is testable. Several synthetic, cell-permeable IAP antagonists have been developed, and they are described to trigger caspase activation and apoptosis in transformed lines. If these similarly trigger apoptosis in cytochrome c mutant cells (but not, say, in APAF-1 null cells), this would provide support for the above model. However, in such a setting, inhibition of caspases by pharmacologic inhibitors should preserve clonogenic survival of the cells since the ensuing death of the cells is predicted to be completely dependent on caspase activation. This prediction of the effects of caspase inhibitors on cellular survival in the presence of IAP antagonists (even in wild-type cells) remains to be evaluated.

However, before we delve too far into these speculations it might be useful to sound a note of caution. The idea that APAF-1 is activated in cytochrome c mutant mice does not necessarily prove that APAF-1 is triggered independently of cytochrome c. As noted above, it is likely that cytochrome c binds to the APAF-1 WD to unmask the nucleotide binding site. It remains possible that the K72A mutant of cytochrome c, while having greatly reduced binding to the APAF-1 WD, nevertheless displaces it to some extent, allowing nucleotide access in some situations. Indeed, this mutant cytochrome c has been shown to trigger caspase activation in cell extracts, albeit at higher concentrations than are normally required for wild-type cytochrome c (Abdullaev et al., 2002). Therefore, cells with mutant cytochrome c may activate APAF-1 and caspases in some but not all cases. For example, K72A mutant cells that contain (and release) higher levels of cytochrome c might activate caspases, or if elevated amounts of dATP or ATP (or perhaps other more arcane nucleotides) are present this may compensate for the reduced activity of the mutant cytochrome c. Thus, it may not be necessary to speculate that APAF-1 is activated independently of cytochrome c, but only that the K72A mutant of cytochrome c is able to activate APAF-1 in only some circumstances.

Another fundamentally interesting observation is also reported by Hao et al. (2005). Bone marrow from the cytochrome c mutant animals, transplanted into wild-type recipients, gave rise after a year to a pronounced B and T cell hyperplasia, splenomegaly, and lymphadnopathy. While we do not know if the same phenotype would be seen with long-term transplantation of APAF-1- or caspase-9-defective bone marrow (published accounts, which did not report such effects, examined much shorter time periods [Scott et al., 2004]), the observations lead to an idea of a “trickle” of apoptosis in wild-type lymphocytes that is missing in the mutant animals.

There are only two broad explanations for this phenomenon, both extremely interesting: (1) lymphoid cells that are destined to die via the mitochondrial pathway may undergo MOMP but may sometimes survive and recover proliferative potential, provided that caspases are not induced, or (2) the mutant cytochrome c has a survival activity in the mitochondria that is not shared by wild-type cytochrome c, resulting in enhanced survival upstream of MOMP. The first possibility would be supported by a similar effect in APAF-1- or caspase-9-deficient cells, which have not been fully examined in this way. Alternatively, if this effect is specific to the mutant cytochrome c (and not APAF-1- or caspase-9

null cells), then the second possibility should be considered. Although this mutant appears to have normal electron transport activity (Hao et al., 2005), any "stalling" might ultimately impact on the function of complex II, which has recently been shown to have important tumor suppressor activity by limiting the accumulation of succinate in the cell (Selak et al., 2005). If, in the mutant cells, succinate accumulates (say, in lymphocytes, which depend more on glycolysis than on oxidative phosphorylation for their energy), this may lead to HIF-1α stabilization by succinate-mediated inhibition of the prolyl hydroxylase required for HIF-1α degradation (Selak et al., 2005). HIF-1α, in turn, promotes cellular survival and could promote the accumulation of activated lymphocytes in these mice. Indeed, HIF-1α stabilization has been shown to block activation-induced apoptosis in T cells, and therefore its stabilization would be expected to result in an accumulation of memory T cells as described (Hao et al., 2005). A careful examination of the respiratory activity of mitochondria from cytochrome c mutant mice shows a slight depression in succinate-driven complex II activity (Hao et al., 2005), perhaps supporting this novel idea.

Cytochrome c Release: Double Indemnity

The results from Hao et al. (2005) help to keep cytochrome c center stage in the mitochondrial pathway of apoptosis. Early in the "ten minutes" that concern us here, the proteins of the mitochondrial intermembrane space, including cytochrome c, are released in a sudden, all-or-nothing manner. In general, two nonexclusive models have been proposed for how the process of MOMP proceeds. In one, proapoptotic members of the Bcl-2 family act to create discontinuities (perhaps pores) in the mitochondrial outer membrane, without affecting the functions of the inner membrane or matrix. In the second, a variety of signals (Ca^{2+}, reactive oxygen species, and many others) trigger the opening of small channels in the mitochondrial inner membrane, allowing water to enter and swell the matrix, effectively bursting the outer membrane, an effect called the mitochondrial permeability transition (mPT). Both models have been widely invoked to explain MOMP in different settings, resulting in apoptosis via the mitochondrial pathway.

Two recent papers have greatly refined this view. In both, mice were generated with targeted disruption of cyclophilin D, a mitochondrial matrix protein involved in mPT (Baines et al., 2005; Nakagawa et al., 2005). As expected the mitochondria from these mice were completely resistant to the induction of mPT by a range of agents. Nevertheless, induction of MOMP by the proapoptotic Bcl-2 family members Bax and Bid (discussed in more detail below) proceeded normally (Baines et al., 2005; Nakagawa et al., 2005). Further, and perhaps more impressively, all forms of apoptosis in a range of cells types, as well as all developmentally controlled apoptosis, occurred identically to that seen in wild-type cells from various tissues (Nakagawa et al., 2005). Thus, mPT appears to be irrelevant for the engagement of the mitochondrial pathway of apoptosis. This conclusion, while valid as a first approximation, can only be formally drawn for those forms of mPT that are depedent on cyclophilin D.

Notably, however, not all cell death in these mice proceeds normally. Another significant form of cell death, characterized as necrosis (based on morphology and lack of an effect of caspase inhibitors on the tempo and mode of death), such as that induced by high doses of hydrogen peroxide, did not proceed in these cells. Strikingly, these mice were remarkably resistant to ischemia/reperfusion injury in the heart (Baines et al., 2005; Nakagawa et al., 2005). The unavoidable conclusion is that cyclophilin D-dependent mPT is fundamental to a mitochondrial pathway of necrotic cell death.

The human intellect is drawn to dichotomies: the mitochondrial pathway of apoptosis involves an mPT-independent MOMP (see below), while the mitochondrial pathway of necrosis depends on and is caused by an mPT. However, things may not be so simple. The mitochondrial pathway of apoptosis is dependent on the proapoptotic Bcl-2 family proteins Bax and Bak (so called "multidomain" or BH123 proteins, see below), and the antiapoptotic Bcl-2 family members (such as Bcl-2 itself) block MOMP and this mitochondrial pathway. But Bcl-2 has also been shown to suppress cell death in the same heart ischemic/reperfusion model used in the cyclophilin D-knockout studies. Therefore modifiers of Bcl-2 function (such as the proapoptotic Bcl-2 family members) might impact on this mode of cell death. (However, ischemic injury elicits a surrounding apoptosis that might be the true target of Bcl-2 in this setting, and therefore this is by no means proof of an effect of Bcl-2 on a mitochondrial pathway of necrotic death dependent on cyclophilin D). Further, mPT-induced MOMP results in the release of cytochrome c (and all other intermembrane space proteins) and can therefore engage the mitochondrial pathway of caspase activation. That this was not observed with high-dose hydrogen peroxide may be a consequence of the direct inhibitory effects of oxygen on the active site of caspases. "Grey zones" may thus exist where an mPT that is insufficient to engage necrotic death may nevertheless result in apoptosis. Future studies using these cyclophilin D-defective animals may help to define these "grey zone" deaths.

With respect to apoptosis, the process of MOMP therefore apparently depends entirely on the function of Bax and Bak to disrupt the barrier function of the outer mitochondrial membrane. Early notions that this was a function of the levels of Bax/Bak versus antiapoptotic proteins (e.g., Bcl-2, Bcl-xL, Mcl-1) envisioned a "rheostat" where death is controlled by changing the levels (and or functions) of the antiapoptotic Bcl-2 family members. Such inhibition of antiapoptotic activity is mediated by the BH3-only subfamily of the Bcl-2 proteins, which share only the third Bcl-2 homology domain with other family members. These bind to the antiapoptotic Bcl-2 proteins and neutralize their activity.

Recent studies by two groups add complexity to this simple rheostat model (Chen et al., 2005; Kuwana et al., 2005). In the first, a survey of the BH3-only proteins and their interactions with several antiapoptotic Bcl-2 family proteins revealed a specificity of interactions where some BH3-only proteins interact with only a subset of antiapoptotic proteins (Chen et al., 2005). Inhibiting multiple antiapoptotic Bcl-2 proteins appears to be required for apoptosis, such that death is controlled by the pattern of functions of these antagonistic players.

However, is the neutralization of antiapoptotic Bcl-2 family proteins sufficient for apoptosis? Bax in its na-

tive, cytosolic, and monomeric form is not constitutively active but must be activated if it is to permeabilize the mitochondrial outer membrane and cause apoptosis (Kuwana et al., 2005). (It is presumed, but not proven, that Bak shares this requirement.) The activation of Bax can be triggered by BH3-only proteins, but again, there appears to be specificity in this interaction. Of those examined, only Bid and Bim were observed to have this function, leading to their designation as "activator" BH3-only proteins. Others (Bad, Puma, Noxa, Bmf, Hrk) did not but instead appeared to perform their function as suggested above, through neutralization of antiapoptotic Bcl-2 proteins. These "derepressor" BH3-only proteins were seen to sensitize cells for apoptosis while not being directly toxic on their own. The emergent view is one of Bax/Bak activation by activator BH3-only proteins that is antagonized by antiapoptotic Bcl-2 proteins and in turn promoted by derepression by other BH3-only proteins.

Cell Death and Society

There is a fundamental problem with multicellular life; selection for cell survival in proliferating cells can be antithetical to the survival of the individual. In the evolution of multicellular individuality, the selective pressures on the cell lineages had to be subsumed in the interest of organismal integrity (Buss, 1987). This problem is solved by how our cells are "wired"; survival of a cell is dependent on the availability of factors produced by other cells, often belonging to distinct lineages. This social interaction among cells ensures that each lineage tends to be held in check, the default being death (Raff, 1992).

This idea, that death is a default (which appears to be at least superficially true at the cellular level), may not be true at the molecular level. In the absence of signals to trigger the activation of Bax and Bak, apoptosis via the mitochondrial pathway will not occur. Indeed, cells lacking Bax and Bak when deprived of growth and survival factors, persist for long periods of time, fueling their metabolic needs through autophagy (Lum et al., 2005). In theory, the same would probably be true of cells lacking signals to activate Bax and Bak. These signals are probably not restricted to the activator BH3-only proteins, but at this point we do not know how many different types of "activators" there are. The alternative idea, that Bax and Bak are constitutively active and are simply held in check by the antiapoptotic Bcl-2 family proteins (the "rheostat" model in its simplest form), is not supported by biochemistry—Bax and Bak are not found in association with antiapoptotic Bcl-2 family members in healthy cells. Apoptosis via the mitochondrial pathway only happens when a positive signal to activate Bax and/or Bak occurs and proceeds without inhibition.

Therefore, in the absence of activation of Bax/Bak it is life, not death, that is the cellular default, and cells will catabolize themselves in order to survive. It follows, then, that the cellular society that is multicellular life is sustained to some extent by the activation of Bax and Bak under conditions of limiting survival signals. Deprivation of survival factors actively generates a trigger for Bax and Bak activation, rather than simply removing the antiapoptotic functions of Bcl-2 and related molecules.

These considerations do not appear to include the mitochondrial pathway of necrotic death, which is not engaged by deprivation of survival factors and does not appear to play a role in normal development (Baines et al., 2005; Nakagawa et al., 2005). Perhaps this is not surprising since this form of cell death is potently inflammatory, as evidenced (for example) by the effects of ischemic injury. In contrast, mPT-less MOMP that is mediated through the actions of Bcl-2 family proteins triggered in the course of cellular socialization (e.g., growth factor deprivation) does not induce inflammation and therefore silently participates in normal development and homeostasis.

These are emerging views of the "ten minutes" in the death of a cell that include MOMP, cytochrome c-induced activation of APAF-1, and caspase activation—ten minutes that can occur at any time following the induction of apoptosis by a stress or other death signal but that probably condemn the cell to its demise. What determines when and if a cell will face these crucial ten minutes remains murky, and we simply do not know in most cases why MOMP and its consequences occur at a particular time hours (or days) after a cell is triggered to die. As we refine our understanding of this critical event, however, we reach new levels of appreciation of the delicate balance that is cellular survival in the complex multicellular individual.

Acknowledgments

Dedicated to the memory of Stanley J. Korsmeyer.

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