PERSPECTIVES: APOPTOSIS

Death of a Monopoly?

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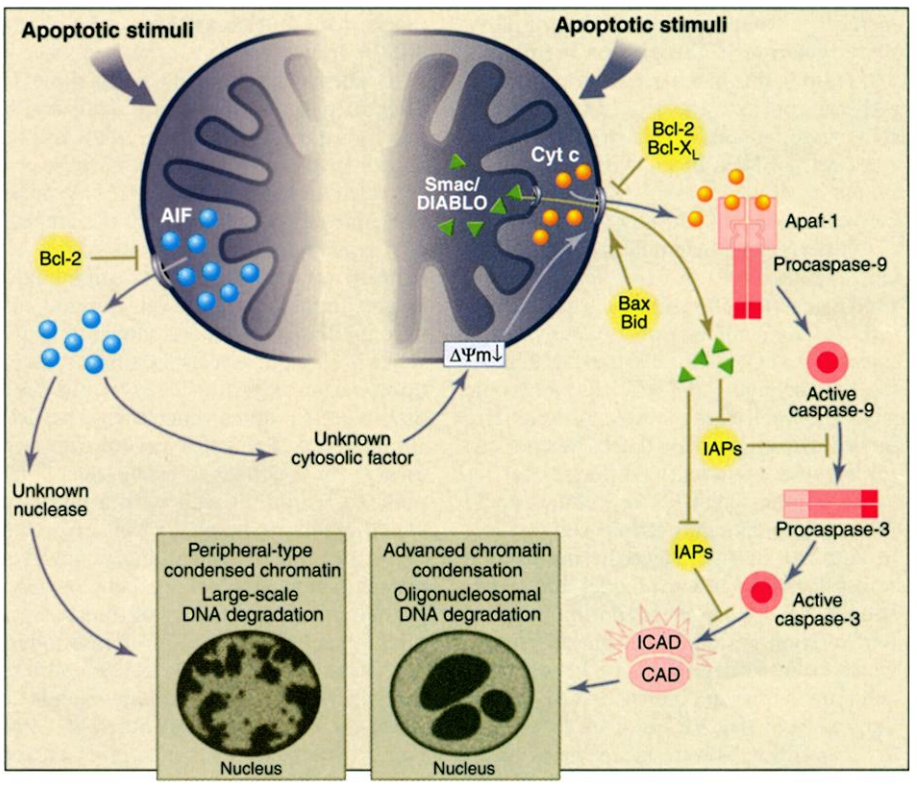
Programmed cell death (PCD, also called apoptosis or physiological cell death), a common and evolutionarily conserved property of all metazoans (*1*), is an essential part of life. In many fundamental biological processes such as development and the strictly regulated cellular homeostasis, PCD is required to eliminate unwanted and supernumerary cells. Thanks in part to genetic studies in the worm *Caenorhabditis elegans*, some aspects of the molecular mechanisms that underlie these crucial cellular events have been unraveled (*2*). We know that a group of cysteine proteases named caspases are central to the death machinery inherited by all cells. Caspases act as the final executioners in a stereotyped cascade of molecular events leading ultimately to the cells' demise (*3*). Although caspases are needed for the complete manifestation of apoptotic morphology and are indispensable for PCD in many systems, they may not always be required, and apoptotic pathways therefore may be more complex and diverse than previously thought. Now, in an article published in *Nature* (*4*), Joza and colleagues report physiological evidence for an alternative mechanism by which cells can undergo apoptosis. They show that a mitochondrially localized factor previously termed apoptosis-inducing factor (AIF) can induce PCD in mammalian cells in a caspase-independent manner, and that this apoptotic pathway is strictly required for early development in the mouse.

Although our understanding of the detailed signaling pathways that can trigger apoptosis is far from complete, tremendous progress in the past 10 years has revealed much about the underlying mechanisms. To date, it is well accepted that diverse apoptotic stimuli converge on a common apoptotic pathway consisting essentially of effector molecules (caspases), adaptor molecules (Apaf-1), and regulatory molecules [pro- and anti-apoptotic Bcl-2 family members, inhibitors of apoptosis (IAPs), and Smac/DIABLO] (see the figure). In this intricate, but well-organized, system the mitochondrion is an integrator of the cell death machinery. In response to multiple apoptotic signals of different origins, the outer mitochondrial membrane is permeabilized, resulting in the release to the cytosol of molecules (for example, cytochrome c, Smac/DIABLO) that are crucial for the activation of downstream effectors of apoptosis. Once caspases are activated, they cleave proteins that will ultimately lead to the morphological manifestations of PCD, such as DNA condensation and fragmentation, and membrane blebbing.

Given the essential role of caspases in the final execution of the cell death program, it would seem logical that disruption of these proteases or key factors regulating their activation (such as cytochrome c or Apaf-1) would have devastating biological consequences, for instance during development when PCD is indispensable. The characteristics of various caspase-deficient mice show that this is indeed partly true, as embryogenesis is frequently compromised in these animals, resulting in prenatal or postnatal lethality [reviewed in (*5*)]. Nonetheless, the observed developmental defects are often confined to certain tissues, suggesting that some forms of PCD, perhaps those acting in the normal parts of the animal, may not necessarily be caspase dependent. Although in some of these mice other caspases are likely functioning to compensate for their missing family members (*6*), another tantalizing



The caspase-dependent and AIF-dependent apoptotic pathways. (Right) Upon apoptotic stimulation, permeabilization of the mitochondrial outer membrane allows the release of cytochrome c into the cytosol, where it induces, together with the adaptor molecule Apaf-1, dimerization and activation of the caspase-9 proenzymes. Active caspase-9 subsequently activates caspase-3, one of the main effector caspases, which in turn cleaves several cellular targets including the inhibitor of the caspase-activated deoxyribonuclease (ICAD), thereby leading to deinhibition of CAD and nuclear apoptosis characterized by advanced chromatin condensation and oligonucleosomal DNA degradation. The caspase-dependent apoptotic pathway is subject to multiple regulatory systems. These include the proapoptotic (Bax, Bid) and antiapoptotic (Bcl-2, Bcl-X_L) Bcl-2 family members that promote or block cytochrome c release, respectively; the inhibitors of apoptosis (IAPs) that block caspase catalytic activity; and the mitochondrial protein Smac/DIABLO that relieves IAPs' inhibition. **(Left)** Mitochondrial AIF released in the cytosol upon apoptotic stimulation can trigger caspase-independent apoptosis through an as yet unknown nuclease. In certain circumstances, AIF can act upstream of cytochrome c release through an unknown cytosolic factor, thereby regulating the caspase-dependent apoptotic pathway (*7*).

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explanation for these phenotypes is the existence of alternative caspase-independent apoptotic pathways.

Just such a pathway was proposed a few years ago when Kroemer's group identified a protein, AIF, capable of inducing DNA condensation and fragmentation of isolated nuclei, and whose activity was not abrogated by the pan-caspase inhibitor z-VAD.fmk (*7*). AIF is a 57-kD flavoprotein bearing both mitochondrial and nuclear signal sequences. Normally confined to the intermembrane space of the mitochondria, AIF translocates to the nucleus upon apoptotic stimulation and induces large-scale chromatin fragmentation by a mechanism that is still unresolved (see the figure). Through a series of elegant in vitro experiments, it was further shown that AIF can induce not only the nuclear hallmark of apoptosis but also many other characteristics of apoptotic cell death, such as dissipation of the mitochondrial transmembrane potential and exposure of phosphatidylserine in the plasma membrane (*7*). Despite this biochemical evidence that AIF can, on its own, trigger apoptosis, it remains to be seen if it is strictly required for a certain form of PCD in vivo, or if it is just a complementary factor along the caspase-dependent pathway.

To definitively examine the physiological function of AIF in vivo, Joza *et al.* (*4*) used a gene-targeting approach to generate AIF-deficient mice. Unfortunately, when injected into C57BL/6 blastocysts, none of the homozygous AIF-deficient embryonic stem (ES) cell clones tested were able to induce chimerism. This defect was not due to defective potential to differentiate, because AIF-deficient ES cell clones could fully differentiate into various cell lineages in vitro and in vivo. Nevertheless, using a well-established in vitro model that reconstitutes the cellular events during the peri-implantation period of the blastocysts [in which cultured aggregates of ES cells develop into embryoid bodies (EBs)], the authors noticed that EBs derived from AIF-deficient ES cells were incapable of forming the proamniotic cavity (a phenomenon called cavitation) that normally results from the demise of epiblasts by PCD. Using bromodeoxyuridine incorporation, they showed that the absence of cavitation in AIF-deficient EBs did not result from uncontrolled proliferation of the epiblasts, but rather from a failure of these cells to undergo apoptosis (as demonstrated by the absence of morphological hallmarks of PCD by electron microscopy). Joza *et al.* also found that neither genetic deficiency of Apaf-1 or caspase-9 nor treatment of wild-type EBs by z-VAD.fmk could reproduce such a developmental defect, suggesting that PCD in the inner cell mass during cavitation proceeds independently of caspase activation. It should be emphasized, however, that although caspase activation was not required for epiblast cell death, it was needed for advanced chromatin condensation: Epiblasts from Apaf-1– or caspase-9–deficient EBs displayed only peripheral chromatin condensation, a feature identical to what is seen in cells microinjected with recombinant AIF (*8*). This latter observation, together with the fact that AIF was required for cytochrome c release and caspase-3 activation in the inner cell mass (*4*), suggests a previously unrecognized sequence of events in which AIF acts upstream of cytochrome c release, thereby regulating the apoptosome-based apoptotic pathway.

Although Joza *et al.* provide strong evidence for a role of AIF during the first manifestation of morphogenetic cell death in mammalian development, their work raises many questions. First, why should AIF be indispensable for the first wave of PCD during development when the main effector caspases (caspases 3, 6, and 7) are expressed at this time (*9*) and, as shown in the present work, caspase-3 is activated during cavitation (*4*)? One possible explanation supported by the authors' data is the apparent specificity of the AIF pathway for certain apoptotic stimuli such as growth factor withdrawal. Because caspase inhibitors have been shown to confer weak protective effects in some systems upon serum withdrawal, it would seem that in certain circumstances caspase activation may not be sufficient (or even necessary) for apoptosis to ensue. Such a hypothesis would fit well with a model in which epiblasts die as a result of growth factor deficiency. Intriguingly, however, growth factors (such as the bone morphogenetic protein subfamily of transforming growth factor β–related proteins) induce cavitation rather than rescuing epiblasts from PCD (*10*). Although one cannot exclude the possibility that other unidentified growth factors might be involved, this raises the question of whether other apoptotic stimuli exist that are specific for the AIF pathway and remain to be revealed. In addition, the requirement for the AIF-dependent apoptotic pathway in response to serum withdrawal (*4*) is puzzling because other groups have reported that cells deficient in cytochrome c, which abrogates the caspase-9/Apaf-1–dependent apoptotic pathway, are also resistant to such apoptotic stimuli (*11*). Finally, the inability of z-VAD.fmk to inhibit PCD during cavitation suggests that caspase-independent death could also be explained by compensatory activation of other caspases that could overcome the lack of caspase-9 or Apaf-1 and still allow PCD to proceed. Although the inability of z-VAD.fmk to inhibit PCD during cavitation suggests caspase independence (*4*), it is important to remember that z-VAD.fmk does not inhibit all caspases equally well (for example, it is a poor caspase-2 inhibitor) (*12*) and therefore cannot be used as a definitive proof of caspase-independent PCD.

Second, the regulation of cytochrome c release by AIF will need clarification. Despite the previous demonstration that an as yet uncharacterized cytosolic factor is required for AIF to induce loss of the mitochondrial transmembrane potential and subsequent release of cytochrome c (*7*) (see the figure), it is still not clear how permeabilization of the outer mitochondrial membrane could cause the release of AIF without a concomitant release of cytochrome c. Such a question applies also for Smac/DIABLO, another mitochondrially localized protein released into the cytosol during apoptosis.

Third, it is important to learn how AIF mediates its apoptogenic properties. Identification of its nuclear and cytosolic targets would shed some light on why it can trigger most of the hallmark features of apoptosis independently of caspases.

Finally, because caspase deficiencies are associated with autoimmunity (*13*) and tumorigenesis (*14*), it would be valuable to know whether the AIF-dependent apoptotic pathway is implicated in pathological circumstances.

Unlike caspases, AIF homologs are found in all three metazoan kingdoms (animal, plant, and fungi), suggesting that AIF could be one of the most ancestral death effectors known (*15*). The findings of Joza *et al.* therefore not only establish the physiological relevance of such an apoptotic pathway in vivo but also raise some exciting questions about the origins of cell death and how it could have evolved to its present form.

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