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Review

Apoptogenic factors released from mitochondria ☆

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ABSTRACT

When cells kill themselves, they usually do so by activating mechanisms that have evolved specifically for that purpose. These mechanisms, which are broadly conserved throughout the metazoa, involve two processes: activation in the cytosol of latent cysteine proteases (termed caspases), and disruption of mitochondrial functions. These processes are linked in a number of different ways. While active caspases can cleave proteins in the mitochondrial outer membrane, and cleave and thereby activate certain pro-apoptotic members of the Bcl-2 family, proteins released from the mitochondria can trigger caspase activation and antagonise IAP family proteins. This review will focus on the pro-apoptotic molecules that are released from the mitochondria of cells endeavouring to kill themselves. This article is part of a Special Issue entitled Mitochondria: the deadly organelle.

1. Introduction

When Kerr, Wyllie and Currie first proposed the apoptosis concept in 1972, they noted that in apoptosis, as opposed to necrosis, the mitochondria did not appear abnormal until late in the process [1]. However, as study of physiological cell death progressed from the era of morphology to the era of biochemistry and molecular biology, the role of the mitochondria in cell death had to be reassessed.

The idea that mitochondria might play a key role in cell death was first raised by David Hockenbery, who was working with Stanley Korsmeyer [2]. They were studying the localization of the cell death inhibitor Bcl-2, the first component of the cell death mechanism to be recognised [3,4]. Although earlier analysis by Cleary et al. had shown that Bcl-2 associates with intracellular membranes via its hydrophobic carboxy-terminus, with the main body of the protein in the cytosol [5,6], Hockenbery et al. concluded that Bcl-2 was an integral protein of the inner mitochondrial membrane [2]. Subsequent work has vindicated Cleary's initial findings [7], and shown that the fraction of Bcl-2 that associates with mitochondria is bound to the outer, rather than the inner membrane. Nevertheless, their paper drew cell death researchers' attention to the mitochondria for the first time.

In an effort to determine what mitochondrial function might be required for apoptosis, Jacobson et al. transfected Bcl-2 into normal cells, and those lacking mitochondrial DNA, and whose mitochondrial were therefore incapable of generating energy by oxidative phosphorylation [8]. Because Bcl-2 was able to inhibit death of both types of cells, as well as rho zero cells cultured in anoxic conditions, they concluded that neither apoptosis nor Bcl-2's protective function required mitochondrial respiration.

In 1994, Newmeyer et al., who were studying apoptosis in a cell-free system derived from *Xenopus* oocytes, found that a mitochondrial factor was necessary for activation of the endonuclease that cleaves nuclear DNA during apoptosis [9]. Furthermore, as release of this factor could be prevented by Bcl-2, their results suggested Bcl-2 acted somewhere upstream of the mitochondria, and the mitochondrial factor(s) were needed for activation of the endonucleases responsible for the DNA "ladders" used as a marker of apoptosis.

Soon after, Kroemer's group reported that when lymphocytes were given an apoptotic stimulus, they lost the electrical potential across the inner mitochondrial membrane, and this occurred prior to cleavage of the genomic DNA [10].

Like Newmeyer, Wang's group were using cell free systems to look for molecules that could activate the apoptotic endonucleases. They found that cytochrome *c* (cyt *c*), which in healthy cells resides in the mitochondrial inter-membrane space, was, following an apoptotic stimulus, released into the cytosol where it triggered activation of the endonucleases [11]. Their subsequent work elucidated the biochemical details of the pathway. They revealed that Bcl-2 could prevent loss of cyt *c* from the mitochondria, but once in the cytosol, cyt *c* bound to the adaptor protein Apaf-1. This caused activation of caspase 9 and caspase 3, which cleaved the endonuclease inhibitor ICAD, thus freeing CAD (Caspase-Activated DNAse), which cleaved the genomic DNA [12–15].

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2. Pro-apoptotic proteins released from the mitochondria

2.1. Cytochrome c

In healthy cells, cyt *c* exists in the mitochondrial inter-membrane space where it acts in the electron transport chain to transfer electrons from complex III to complex IV. In the cytoplasm of cells undergoing apoptosis, cyt *c* binds to Apaf-1, which binds to caspase 9, thus forming the apoptosome [16,17]. Each cytochrome *c* polypeptide binds a haem molecule, which is necessary for it to carry electrons, and is also required for cyt *c* to activate Apaf-1 [12,18].

By binding to Apaf-1 and triggering formation of the apoptosome, presence of cyt *c* in the cytosol is *sufficient* to cause caspase-dependent cell death, but is it *necessary* for cells to die by the mechanism that bcl-2 can block? Two sets of experiments suggest that this is not the case, and suggest that cell death occurs even when cyt *c* cannot bind Apaf-1. They show that although cyt *c* and Apaf-1 are potent caspase activators, and can efficiently cause cell death, neither are essential for cell death by the mechanism Bcl-2 can block.

Firstly, cells cultured in anoxic conditions cannot synthesize haem, and hence only contain apo-cytochrome *c* [19]. Nevertheless, such cells are still killed by an apoptotic stimulus such as staurosporin, and cell death is still inhibited by Bcl-2 [8]. Presumably, something other than Apaf-1 activated caspases killed the cells. The cells also did not die due to lack of mitochondrial respiration, because these cells were producing ATP by anaerobic glycolysis.

Secondly, genetic experiments in which the genes for Apaf-1 were deleted, or the genes for cyt *c* were modified so that it could no longer bind to Apaf-1 but could still function in mitochondrial respiration, showed that although cell death is somewhat delayed, it still occurs both *in vitro* and *in vivo* when Apaf-1 cannot be activated, or is absent [20–24].

These experiments suggest that although cyt *c* is a molecule that is released from the mitochondria during apoptosis, and can accelerate cell death by binding to Apaf-1 and activating caspases, cyt *c* does not play an essential role in cell death, because cell death occurs normally in mice (of the right genetic background) that lack Apaf-1 or bear a form of cyt *c* that cannot bind to Apaf-1.

2.2. Smac/Diablo

Smac (second mitochondrial activator of caspases)/Diablo (direct IAP binding protein with low pI) was given its alternative names because of the methods used to identify it. Wang's group isolated Smac/Diablo from HeLa cell lysates as a component from solubilized membranes that could enhance activation of caspase 3 *in vitro*, and found that, like cyt *c*, Smac was a mitochondrial protein released into the cytosol in cells undergoing apoptosis [25]. Vaux's group isolated Smac/Diablo as a protein that could be co-immunoprecipitated from cell lysates together with XIAP [26].

By binding, via its N-terminal IAP Binding Motif (IBM), to the baculoviral IAP repeats (BIRs) of XIAP, cytosolic Smac/Diablo was able to displace processed caspase 9 and caspase 3, which were then free to dismantle the cell [27–29]. Indeed, even short peptides corresponding to the IBM were able to promote apoptosis of some tumor lines [30]. Observations such as these prompted pharmaceutical companies to develop synthetic Smac mimetic compounds, to see whether antagonism of IAPs *in vivo* could cause tumor cell death. Although these IAP antagonist compounds can indeed kill certain cancer cell lines, and several have entered clinical trials, questions remain about the functions of, and requirements for, Smac/Diablo in both healthy cells and those undergoing apoptosis.

For example, gene knockout experiments in mice have not given much support to the notion that Smac/Diablo plays import roles either in healthy cells or cells undergoing apoptosis. Mice lacking Smac/Diablo developed normally, and were fertile. Moreover, no abnormality could be detected in any cell type, no matter what stimulus was used [31]. Similarly, mice mutant for XIAP showed no overt phenotype, and exhibited no change in sensitivity to apoptotic stimuli [32]. The simplest explanation for failure to detect a phenotype in these mice is that both Smac/Diablo and XIAP are redundant, presumably with other IAP binding proteins, and other IAPs, respectively. What are the other IAP binding proteins?

2.3. HtrA2/Omi

The co-immunoprecipitation experiments that allowed identification of Smac/Diablo as an XIAP binding protein also identified other proteins [26,33,34]. In each case, the IAP-binding proteins were mitochondrial proteins, and all bore an IBM that allowed them to bind to the BIRs of IAPs. One of them, HtrA2/Omi, was also identified by other groups by virtue of its ability to bind to IAPs [35–37].

In the mitochondrial intermembrane space, HtrA2/Omi acts as a chaperone and serine protease that re-folds or degrades misfolded mitochondrial proteins, much like its bacterial homolog, HtrA, does in *Escherichia coli* [38]. Bacterial HtrA (also known as DegP) forms a hexameric complex [39] that functions in a way reminiscent of the proteasome in eukaryotic cells.

Deletion of the genes for HtrA2/Omi in mice did not cause resistance to cell death, but resulted in development of a neurodegenerative disease that caused death by 30 days. This was presumably due to accumulation of misfolded proteins in the mitochondria of long-lived neuronal cells [40]. Furthermore, mice with deletion of both Smac/Diablo as well as HtrA2/Omi genes had the same phenotype as those lacking just HtrA2/Omi, providing no support for the hypothesis that Smac/Diablo and HtrA2/Omi have redundant functions [40].

Over-expression of HtrA2/Omi does have some pro-apoptotic activity, such as the ability to increase the amount of UV induced apoptosis of a cell line transfected with XIAP, but it was weaker than Smac/Diablo, and it was still able to do so, albeit to a reduced extent, even if its IBM was mutated. Together with the results of the genetic experiments [40,41], these results raise the possibility that increased sensitivity to apoptosis caused by over-expression of HtrA2/Omi is due to presence of a serine protease in the cytoplasm, rather than HtrA2/Omi having a physiological role as an IAP regulator [33,36,37].

2.4. Other mitochondrial IAP binding proteins (Nipsnap3 & 4, glutamate dehydrogenase, ClpX, LRPPR, 3-hydroxyisobutyrate dehydrogenase)

In addition to Smac/Diablo and HtrA2/Omi, Verhagen et al. have identified six further proteins that normally reside in the mitochondria, but bear IBMs and are capable of binding to IAPs if released into the cytosol [34]. Although it is possible that they can antagonize IAPs, and do so in Smac/Diablo and HtrA2/Omi mutant cells, thereby allowing them to undergo apoptosis, this seems unlikely. Most of these proteins have some other, well established role in the mitochondria, and none show significant pro-apoptotic activity when expressed in the cytoplasm. An alternative possibility is that in mammalian cells there are IBM binding proteins that are produced and reside in the cytosol, just as the IAP antagonists Reaper, Grim, HID and Sickle do in insect cells [42,43].

3. Other apoptogenic proteins released from mitochondria

There is evidence that a number of other pro-apoptotic proteins are released from the mitochondria, but in general the evidence is weak and inconsistent.

3.1. Apoptosis inducing factor (AIF)

AIF was initially thought to be a mitochondrial cysteine protease whose release could be blocked by Bcl-2, but following an apoptotic stimulus translocated from the mitochondria to the nucleus to cause DNA fragmentation and cell death [44]. Moreover, it was claimed that AIF was essential for programmed cell death [45], and that in the nucleus it directly bound to DNA [46]. However, more recent genetic and biochemical evidence has shown that AIF is not required for cell death [47], but is a highly conserved oxidoreductase/NADH oxidase that is a component of complex I of the mitochondrial electron transport chain [48,49]. Furthermore, the phenotype of Harlequin mice, which have low levels of AIF, resembles that of mice with deficiencies of complex I [50,51], rather than those with excess cells, which would have been expected if it had a pro-apoptotic function.

3.2. EndoG

By creating transgenic mice that ubiquitously expressed an uncleavable form of ICAD {the Inhibitor of Caspase Activated DNAse (CAD)}, Nagata's group showed that in the absence of CAD activity, DNAse II from cells that engulfed the apoptotic corpses degraded their DNA [52]. While trying to address the same question, Wang's group used a biochemical approach, and identified the mitochondrial endonuclease, endonuclease G (endoG) [53]. They found that endoG was released from the mitochondria during apoptosis, and could enter the nucleus and cleave genomic DNA without being activated by caspases. Although analysis of an endoG knockout mouse initially gave support to this model because cells from these mice were resistant to certain apoptotic stimuli and exhibited reduced DNA cleavage [54], when another group deleted the genes for endoG, but did not also disrupt an adjoining gene, they found no phenotype [55]. Therefore it seems most likely that DNAse II is responsible for DNA cleave when CAD cannot be activated, and even if endoG (and AIF) are released from the mitochondria during apoptosis, they do not cause DNA fragmentation, and the physiological significance of their release, if any, has yet to be established.

3.3. Caspases

There have been a number of reports that caspases, including procaspases 2, 3, 8 and 9 reside in the intermembrane space of mitochondria in healthy cells, and are activated and released during apoptosis [56–62]. However, these claims have not been confirmed, caspases do not appear in comprehensive mitochondrial proteome analyses [63], and a mechanism by which caspase precursors could enter the mitochondria in the absence of targeting sequences has not been elucidated.

3.4. Others

Adenylate kinase 2 (AK2) is a protein that resides in the mitochondrial intermembrane space and can convert AMP, ADP and ATP from one to another [64]. It has been reported that when released into the cytosol, AK2 can bind to FADD and promote activation of caspase 10, but curiously, not caspase 8 [65]. However, mutation of AK2 genes in humans leads to a degenerative disease termed reticular dysgenesis that is consistent with deteriorating mitochondrial function, rather than increased cell survival that would be expected if it had a pro-apoptotic function [66,67].

4. How are mitochondrial proteins released?

Several models have been proposed to explain how proteins are released from the mitochondria during apoptosis. For example, it has been suggested that proteins are released via the permeability transition pore, consisting of ANT, cyclophilin D and VDAC. While this pore is usually too small to allow transit of proteins as large as cytochrome *c*, it has been proposed that pores might fuse, or enlarge in the presence of other proteins. Another model is that entry of ions and water into the matrix causes the inner membrane to swell by osmosis, causing the outer membrane to physically rupture [68,69]. Neither of these models are supported by analysis of cells from Bax: Bak double deleted mice.

Currently the most widely favored model is that in the presence of high levels of pro-apoptotic BH3-only Bcl-2 family members, or low levels of anti-apoptotic Bcl-2 family members, Bak or Bax multimerize to form a pore in the outer mitochondrial membrane [70]. Study of release of tagged proteins from the mitochondria during apoptosis suggest that most are released via the same mitochondrial pore, and by a caspase independent mechanism [71]. However, late in apoptosis, after caspases become active in the cytosol, they can cleave proteins in the mitochondrial outer membrane, causing it to completely break down [72].

No matter what the mechanism by which mitochondrial proteins exit the mitochondria, it is clear that in apoptosis proteins do leave, but debate remains about the significance of this occurrence.

5. (Lack of a) role in apoptosis of proteins released from the mitochondria of invertebrate cells

Invertebrate model organisms such as *Drosophila* and *Caenorhabditis elegans* have accelerated elucidation of many biological processes, not the least of which are the physiological mechanisms for cell death. For example, genetic analysis of cell death during development of *C. elegans* revealed that the caspase CED-3 is essential for programmed cell death in the worm, and it is activated by an Apaf-1 like adaptor protein, CED-4. Moreover, the Bcl-2-like protein CED-9 is able to prevent CED-4 from activating CED-3. However, in the worm CED-9 binds directly to CED-4 to inhibit it, rather than acting to prevent release of proteins from mitochondria, as Bcl-2 does, by inhibiting Bax and Bak in mammalian cells. Unlike Apaf-1, which has WD40 domains that can be bound by cytochrome-c, *C. elegans* CED-4 lacks WD40 domains, and appears to activate spontaneously when inhibition by CED-9 is removed. Thus, although CED-9 and CED-4 can localize to outer mitochondrial membranes, implementation of the apoptotic process does not require any mitochondrial protein in the worm [73].

Drosophila also bears genes for homologs of Bcl-2, Apaf-1 and caspases, and, like their *C. elegans* homologs, they are essential for normal developmental cell death [74]. Although there were some early reports that insect cytochrome *c* released from the mitochondria was needed to activate the Apaf-1 homolog dARK [75,76], more thorough genetic and biochemical analysis indicates that dARK can activate the caspase DRONC in the absence of cyt *c*, and *Drosophila* cyt *c* does not bind to dARK, even though it has WD40 domains [77,78].

Study of cell death in insects revealed the roles of the IAPs and their antagonists Reaper, HID, Grim and Sickle [79–81]. The IAPs were first identified in baculoviruses, which use them to prevent defensive apoptosis of the host cell, allowing more time for viral replication [82]. Based on their similarity to baculoviral IAPs, their cellular homologs DIAP1 and DIAP2 were identified in the fly [83,84]. Abrams screened for flies with abnormal cell death during development identified a region (H99) required for developmental cell death [79]. Subsequent work revealed that the pro-apoptotic proteins Reaper, HID, Grim and Sickle bind directly to the IAPs via their amino-termini, which resemble those of Smac/Diablo and HtrA2/Omi [85,86]. However, unlike the mitochondrial IAP antagonists, those from *Drosophila* reside in the cytosol, and are therefore not released from the mitochondria.

6. Conclusions

Although it is clear that Bax and Bak are critical mediators of apoptosis that function at the mitochondria, the only mitochondrial

protein for which there is strong evidence that it is released from the mitochondria and plays a role in promoting cell death is cytochrome *c*. It is clear that many other proteins are released at the same time, and presumably via the same route as cyt *c*, but evidence is lacking that any of them play essential roles in cell death, or that prevention of their release will stop any cell from dying. Furthermore, as caspases become active, they can cleave proteins of the outer mitochondrial membrane, leading to secondary release of mitochondrial proteins into the cytosol, but this is presumably a late, post-mortem event from which a cell cannot recover.

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