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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.