Cell Death and Differentiation (2006) 13, 1291–1295

www.nature.com/cdd

News and Commentary

Which came first, the cytochrome *c* release or the mitochondrial fission?

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Cell Death and Differentiation (2006) **13,** 1291–1295.
doi:10.1038/sj.cdd.4401985; published online 9 June 2006

Mitochondria play a crucial role in apoptosis triggered by many stimuli. They integrate death signals through Bcl-2 family members and coordinate caspase activation through the release of apoptogenic factors, such as cytochrome *c*, as a result of the outer mitochondrial membrane becoming permeable. At the same time, mitochondria fragment and their internal ultrastructure is altered. Here, we attempt to summarize current views of the mechanism that leads to mitochondrial fission during apoptosis and the role that this event plays in this process.

Mitochondria are essential for life of eukaryotic cells as they are the main source of energy. Depending on the cell type and the metabolic demands, these organelles can adopt various shapes from long, tubular and filamentous to small and punctiform.[1] Their morphology is dynamically remodeled for a large part by continuous events of fission and fusion. The protein machineries responsible for mitochondrial fusion and fission are described in Box 1. When fusion events overcome fission events, then mitochondria are elongated whereas they are punctiform when fission events are predominant. In most cell types, including primary and transformed cells, mitochondria are predominantly elongated and densely packed in the perinuclear region. However, in primary hepatocytes, they are punctiform and evenly distributed within the cell.[2]

Mitochondria are also essential for apoptosis triggered by many stimuli. When cells undergo apoptosis, the morphology of mitochondria changes resulting in small, round and more numerous organelles. The observation that the morphology of mitochondria is altered during cell death is not new. More than 50 years ago, well before the seminal description of apoptosis by Kerr *et al.*,[3] Roullier[4] observed a reduction in mitochondrial volume during cell death. This phenomenon was not reported by Kerr *et al.*[3] who may not have been able to determine in detail the length of mitochondria winding through a cell in their thin section ultrastructural studies of apoptotic cells. The reduction in mitochondrial volume during cell death and in particular during many apoptotic responses involving Bcl-2 family members was re-discovered recently in various cell types[5–10] and in various species, including *Caenorhabditis elegans*[11] and trypanosomes.[12] Since then, the identification of the molecular mechanisms responsible for the remodeling of mitochondria during apoptosis and the study of the physiological relevance of this process in cell death have been the matter of intense research. As in so many areas of cancer biology and cell death research, Stan Korsmeyer played a pioneering role on this topic[13] and his work suggests the model proposed in Figure 1 as discussed later in detail.

The aim of this review is to summarize these findings and to address several key questions: Is the change in mitochondrial volume occurring during apoptosis due to a fission of the organelle? What is the molecular mechanism underlying this event? When does it occur relative to outer membrane

Box 1 Proteins involved in mammalian mitochondrial fission and fusion ([1])

The mitochondrial fission machinery
Drp1 and Fis1 mediate mitochondrial fission in mammalian cells. Fis1 is anchored to the outer mitochondrial membrane by a single hydrophobic domain with two tandem tetratrico-peptide repeat motifs facing the cytosol, which mediate protein–protein interactions between Fis1 and other proteins.[46,47] Fis1 is thought to be a receptor for Drp1. Drp1 is a large cytosolic GTPase with similarities to dynamin.[48,49] The protein is cytosolic and translocates to mitochondria where it couples GTP hydrolysis to fission of the organelle by a mechanism that is not completely understood.
In addition to these proteins, MTP18[50] and GDAP1[51,52] have been found to be required for mitochondrial fission in mammalian cells.

The mitochondrial fusion machinery
The dynamin-family members, OPA1 (Optic Atrophy 1) and MFN1 and 2 (Mitofusins) are required for mitochondrial fusion in mammalian cells.
Mitofusins 1 and 2 are integral proteins of the outer mitochondrial membrane that form homo and hetero-complexes with each other. The N-terminal GTPase domain of MFN1 is required for fusion activity and is orientated towards the cytosol. The C-terminal coiled-coiled domain also faces the cytosol where it coordinates the docking of mitochondria to one another through antiparallel binding to the C-terminal coiled-coil domains of MFN1 or MFN2 molecules on adjacent mitochondria.[53] Deletion of mitofusins 1 or 2 genes is embryonically lethal in mice.[43] Moreover, mutations in MFN2 are associated with Charcot-Marie-Tooth type 2A, an inherited peripheral neuropathy.[54,55] OPA1 is a large GTPase localized to the inner mitochondrial membrane facing the intermembrane space. Several isoforms of OPA1 (at least eight) have been identified. Their role in the control of the fusion of the inner mitochondrial membrane is not clearly understood. Mutations in OPA1 have been found to be responsible for dominant optic atrophy, the most common form of hereditary blindness, which is due to the degeneration of ganglion cells of the retina.[56,57]

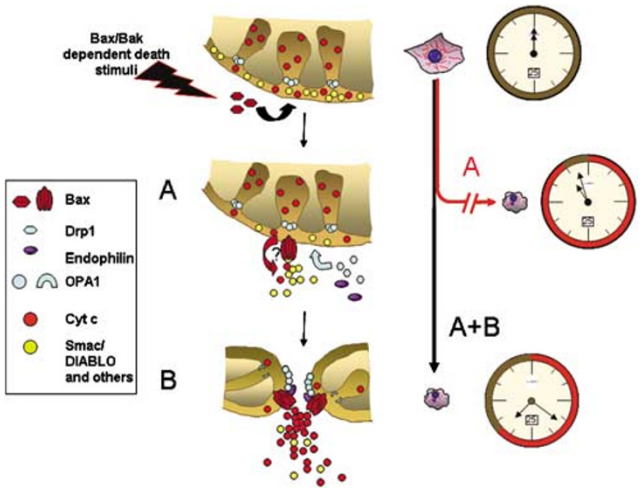

Figure 1 Mitochondrial fission together with cristae remodelling contribute to the dynamics of cytochrome *c* release. Left: Upon induction of apoptosis by many Bax/Bak-dependent death stimuli, Bax translocates to the OMM and oligomerizes at sites of future mitochondrial fission. This leads to MOMP and to the efflux of a small amount of cytochrome *c* as well as of many other proteins that are soluble in the IMS. Concomitantly to MOMP, Drp1, maybe following interaction with DDP/TIMM8a,[30] is recruited to the mitochondria and triggers mitochondrial fission which leads to loss of OPA1 function. This results in opening of the cristae junctions and mobilization of cristae-endowed cytochrome *c* that can be released across the permeabilized OMM. Right: When the release of cytochrome *c* is complete, cell death occurs rapidly (black arrow). Inhibiting mitochondrial fission prevents the release of the major pool of cytochrome *c* that remains sequestered in the cristae. As a consequence, activation of caspases is retarded, offering the cells to survive longer (red arrow)

permeabilization? How does this event participate in apoptosis?

Is Fission Responsible for the Size Reduction of Mitochondria During Apoptosis?

It was initially hypothesized that components of the 'classical' fission and fusion machineries, which allow mitochondria to fragment and fuse continuously, were also responsible for the remodeling of mitochondria during apoptosis.[10] Expression of a dominant-negative mutant of Drp1, DrpK38A, was observed to be sufficient to maintain a normal mitochondrial morphology during apoptosis. Inhibition of the fission machinery with RNAi targeted to Drp1 or hFis1 gave identical results,[14] strongly suggesting that at least some of the components of the classical fission machinery participate actively in shaping mitochondria during apoptosis. Concomitant to the fission machinery being activated, the fusion of mitochondria was found to be inhibited, which exacerbates the disintegration of the mitochondrial network.[15] In addition to the alteration of the fission/fusion machineries, it is probable that other events contribute to the fragmentation of the mitochondrial network. In particular, modification of cytoskeleton elements that contribute to provide mitochondria their shape and dynamics could be involved. For example, inactivation of mitochondria-associated kinesin by phosphorylation was found to be responsible for the perinuclear aggregation of mitochondria during TNF-induced apoptosis.[16]

Fissioning the Mitochondria

The changes in mitochondrial shape during apoptosis result from an activation of the fission machinery and concomitant neutralization of the fusion machinery. When, how and why are these events occurring during apoptosis?

When does mitochondria fission occur?

The fission of mitochondria is an early event of many apoptotic responses that involve Bcl-2 family members. It occurs within the same time frame as the activation of Bax and permeabilization of the outer mitochondrial membrane (MOMP) that leads to the release of cytochrome *c* and many other proteins from the intermembrane space (IMS) into the cytosol. Although the precise mechanisms of MOMP are still debated, it is accepted that Bax and Bax-like proteins are required for this process.[17–20] In search for a causality link between MOMP and mitochondrial fission, many researchers have attempted to determine whether mitochondrial fission occurs before, concomitantly or after MOMP.[21–23] The answer to this question is still unclear, probably due to the limits of the techniques used to resolve temporally events that are almost coincident. Cytochrome *c* filled fragmented mitochondria have been observed frequently during the apoptotic process, suggesting that mitochondrial fission occurs before MOMP. However, it is impossible to exclude that those mitochondria had started to release amounts of cytochrome *c* too low to be detected. On the other hand, it is very rare to find elongated mitochondria that are depleted of cytochrome *c* during apoptosis. In agreement with Gao *et al.*,[23] we have counted less than one in 1000 cells undergoing apoptosis containing elongated mitochondria that were negative for cytochrome *c* immunostaining (Parone *et al.*, unpublished data). At first view, this would be another argument in favor of mitochondrial fission preceding or being concomitant to MOMP. However, if we consider that the time lapse between MOMP and mitochondrial fission is very short, then even a small number of cytochrome *c* depleted filamentous mitochondria could be consistent with mitochondrial fission following MOMP. In addition, it is clear that the fission of mitochondria *per se* does not seem to be necessary for MOMP as there are many cell types in which mitochondria are constitutively fragmented and in which MOMP and apoptosis occur normally when the cells are exposed to death stimuli. This is the case, for example, for the MFN1 or 2-depleted MEFs in which mitochondria, although fragmented, undergo MOMP normally after staurosporine or actinomycin D treatment (D James, unpublished data). It is also possible to reversibly fragment mitochondria with uncouplers or other drugs, showing that fragmentation need not cause apoptosis.[24,25] Rather than the morphology of mitochondria *per se,* it appears that what is important is the mitochondrial recruitment, or the nonrecruitment, of proteins involved in the fission and fusion of the organelle during cell death. So rather than looking at the morphology of

mitochondria and MOMP, it could be more instructive to study how proteins of the fission machinery are recruited to mitochondria relative to Bax activation.

How does mitochondrial fission occur?

What are the molecular mechanisms responsible for activation of the fission machinery and for the concomitant inhibition of the fusion machinery? How can these two events be coregulated during apoptosis?

The answer to these questions is still evasive, particularly because we are still ignorant of how the fission and fusion of mitochondria are regulated in healthy cells. Bax or Bax-like proteins could be directly involved in the activation of Drp1. Immediately upon Bax translocation (within the current time limits of resolution of seconds), Bax and Drp1 localize together in discrete foci on mitochondria at prospective scission site.[26] However, no direct interaction between these proteins has been reported. Moreover, Drp1 was found to be normally recruited to mitochondria in Bax/Bak-deficient MEFs exposed to various death stimuli (mentioned as data not shown in Sugioka *et al.* 2004[27]). Endophilin B1/Bif1, a protein that interacts with Bax only upon induction of apoptosis, has also been proposed to participate in the activation of mitochondrial fission.[28] However, recent data suggest that Endophilin B1/Bif1 acts upstream of mitochondrial fission by triggering Bax and Bak activation.[29] Incidently, Arnoult *et al.*[30] found that OPA1 and DDP/TIMM8a are both released from the mitochondria after MOMP during Bax/Bak-dependent cell death. Whereas the release of OPA1 could potentially explain the inhibition of mitochondrial fusion, binding of DDP/TIMM8a to Drp1 would promote Drp1 recruitment to the mitochondria. This would provide a mechanism for a coordinate regulation of mitochondrial fission and fusion during apoptosis. Nevertheless, this model still does not explain how Drp1 is recruited to mitochondria in Bax/Bak-depleted cells exposed to death stimuli, as MOMP does not occur in those cells.[27]

Why mitochondrial fission?

Inhibition of proteins of the fission machinery have been reported to influence either Bax activation and MOMP or only the degree of MOMP. Translocation of Bax and exposure of its N-terminal domain (which corresponds to part of its activation) have been shown to occur normally in cells depleted of Drp1 or transfected with DrpK38A.[10] This is contrary to results obtained by Neuspiel *et al.*[31] who showed that the amount of conformationally active Bax at the mitochondria in the early stages of apoptosis is significantly reduced in DrpK38A-transfected cells. Furthermore, overexpression of rat Fzo1A,B,[27] or elimination of hFis activity by RNA interference[14] also delays the translocation of Bax to the mitochondria and the oligomerization of Bax and Bak at the MOM. It is still not clear how preventing mitochondrial fission during cell death affects the translocation of Bax and the activation of Bax/Bak at the MOM. It is possible that different components of the mitochondrial fission/fusion machinery are required at distinct steps of the activation of Bax/Bak. The fused morphology of the mitochondria might hamper the insertion of Bax/Bak in the MOM. This would be consistent with previous data showing that membrane curvature influences the permeabilization of liposomes by Bax.[32,33]

By which mechanism could inhibition of mitochondrial fission decrease MOMP, despite normal Bax activation? Previous studies have shown that two pools of cytochrome *c* exist within the mitochondria: a minor pool that is soluble in the IMS and a major pool confined to the mitochondrial cisternae.[13,34,35] Furthermore, electron microscopic tomography of mitochondria has revealed that the IMS is separated from the intra-cisternal space by narrow cristae junctions.[36] Therefore, to account for the complete and fast release of cytochrome *c* during apoptosis, cisternal cytochrome *c* must be relocated into the IMS before it can be released across a permeabilized OMM. MOMP would only allow the release of proteins that are soluble in the IMS. Consistent with this hypothesis, inhibiting the mitochondrial fission machinery does not completely block the release of cytochrome *c* and does not prevent the release of SMAC (Parone *et al.*, submitted). Recently, it has been shown that, during apoptosis, concurrent with their fission, mitochondria undergo ultrastructural changes that include disruption of cristae junctions, opening of the cristae and expansion of the IMS.[13,37] OPA1 is a natural candidate to control remodeling of the intra-mitochondrial space during apoptosis as its loss leads to widened mitochondrial cristae and facilitates cytochrome *c* release.[38,39] The model predicts that mitochondrial fission would lead to a loss of function of OPA1 (Figure 1). It now remains to be tested whether the function of OPA1, which resides on the IMS face of the inner membrane, can be altered during fission of mitochondria and how this could occur.

Mitochondrial fission and cell death

It was reported that inhibiting the fission machinery by overexpression of DrpK38A not only preserved the morphology of mitochondria during cell death but also conferred cell protection against various death stimuli involving proapoptotic members of the Bcl-2 family.[10] Without this result, the fission of mitochondria would have remained a cell death epiphenomenon. A large number of studies have now confirmed a role of mitochondrial fission in cell death.[11,14,27,31] In addition, it was reported that triggering excessive mitochondrial fission, either by overexpression of hFis1, Drp1 or depleting OPA1 was sufficient to induce apoptosis.[11,38,40,41] The mechanism by which apoptosis occurs under these circumstances is not well understood. Fission of mitochondria *per se* is probably not the cause because as already mentioned there are many cell types that can survive normally with punctiform mitochondria. Rather, overexpression of hFis1 or Drp1 or depletion of OPA1 could lead to mitochondrial dysfunction that would represent the intrinsic cause of cell death.

It is important to mention that the cell protection conferred by inhibition of mitochondrial fission is partial and incomplete compared, for example, to the strong protection conferred by antiapoptotic proteins of the Bcl-2 family (Parone *et al.*, submitted). In *C. elegans*, inhibiting mitochondrial fission by overexpression of DrpK38A only rescues 10% of the cells destined to die during development.[11] This animal has also provided the best experiment distinguishing mitochondrial fission from apoptosis. Two mutants have been identified,

Ced-9 (n1950gf) and Ced-9 (n2812lf), that both block mitochondrial fission induced by EGL-1 or Drp1. However, ced-9 (n2812lf) animals, contrary to ced-9/n1950gf animals, are not viable owing to a loss of function in the antiapoptotic activity of this protein. As a result, ectopic developmental programmed cell death that is dependent on both ced-4 and ced-3 occurs and the animals die despite decreased mitochondrial fission. However, these findings are not transposable to mammalian cells because in the nematode MOMP and cytochrome *c* release do not seem to occur during apoptosis. Most intriguing, perhaps, is that there appears to be conservation of the mitochondrial fission process during apoptosis between round worms and mammals, and the connections between the fission machinery and apoptosis may predate the evolution of the cytochrome *c* release step. This is consistent with a recent report that Ced-9 expressed in mammalian cells can induce mitochondrial fusion without inhibiting cytochrome *c* release and apoptosis.[42]

We see two explanations for the incomplete protection conferred by mitochondrial fission inhibition. First, the techniques used (in most cases RNAi approaches) do not completely downregulate expression of the proteins that are targeted and the small amounts that are left could be sufficient to maintain normal activity during apoptosis. Studies on knockout (KO) animals should be better but it is likely that knocking out the genes encoding proteins involved in mitochondrial fission will be lethal (as this is the case for the genes involved in mitochondrial fusion[43]). However, MEFs derived from these animals could be useful. The second explanation is that inhibiting mitochondrial fission (even if we assume that complete KO is obtained for each protein studied) does not lead to a complete inhibition of MOMP and cytochrome *c* release. This would be consistent with mitochondrial fission occurring after MOMP. Mitochondrial fission could be seen as a positive feedback to amplify MOMP and the release of cytochrome *c*. The amount of cytochrome *c* that is initially released, before recruitment of proteins of the fission machinery and mitochondrial fission, would be, in most cells, sufficient to trigger the formation of the apoptosome and caspase activation. Inhibiting mitochondrial fission would only slow down the kinetics of cell death (Figure 1).

The amount of cytochrome *c* that is required to trigger caspase activation is not known. This amount may vary according to different cell types. We postulate that in cells requiring low amounts of cytochrome *c* for caspase activation, mitochondrial fission may not be a necessary event. However, in cells requiring a high amount of cytochrome *c* to activate caspases (maybe cells expressing high amounts of IAPs), the mitochondrial fission could play an important role in stimulating cytochrome *c* release. Of course, this implies that MOMP in those cells is not an irreversible event. Neurons and cardiomyocytes, for example, are able to recover after MOMP. Following growth factor deprivation (NGF), sympathetic neurons can survive for up to 7 days with cytochrome *c*-depleted mitochondria, provided that caspases are inactive.[8] They can recover a normal life if re-exposed to NGF. Cardiomyocytes are resistant to staurosporine-induced apoptosis even though cytochrome *c* is released from mitochondria, likely due to the low level of Apaf-1 in these cells.[44,45] As in neurons, removal of the apoptosis inducer following cytochrome *c* release allows the long-term survival of the cardiomyocytes. Preventing mitochondrial fission in cells could delay caspase activation and allow certain cell types to survive transient stresses until more favorable environmental conditions are restored.

In conclusion, regardless of the sequence of events, mitochondrial fission occurs in a wide variety of cell types using the physiologic organelle scission machinery, upstream of caspase activation, and close in time to cytochrome *c* release. As rapid and efficient catabolism of cells is one important feature of apoptosis, perhaps the mitochondrial fission process is mobilized to expedite the breakdown and removal of the long and interconnected networks. Mitochondrial fission may also be a prerequisite for autophagy of the organelles and the connections between autophagy and apoptosis may also help illuminate how mitochondrial fission is coupled to the apoptosis cascade.

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