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Review

Mitochondrial outer-membrane permeabilization and remodelling in apoptosis

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ABSTRACT

Many human pathologies are associated with defects in mitochondria such as diabetes, neurodegenerative diseases or cancer. This tiny organelle is involved in a plethora of processes in mammalian cells, including energy production, lipid metabolism and cell death. In the so-called intrinsic apoptotic pathway, the outer mitochondrial membrane (MOM) is premeabilized by the pro-apoptotic Bcl-2 members Bax and Bak, allowing the release of apoptogenic factors such as cytochrome c from the inter-membrane space into the cytosol. At the same time, mitochondria fragment in response to Drp-1 activation suggesting that mitochondrial fission could play a role in mitochondrial outer-membrane permeabilization (MOMP). In this review, we will discuss the link that could exist between mitochondrial fission and fusion machinery, Bcl-2 family members and MOMP.

Contents

1. Introduction

Apoptosis is a form of programmed cell death essential for homeostasis, which is frequently dysregulated in human pathologies such as cancer, neurodegenerative diseases or viral infections (Meier et al., 2000; Vaux and Korsmeyer, 1999). In their natural environment, cells are exposed to various stimuli which they can interprete either as “good” or “harmful” and that determine their path towards life and death. In several apoptotic pathways, such an alternative is made at the level of mitochondria. This organelle is permeabilized by the pro-apoptotic proteins of the Bcl-2 family Bax and Bak, leading to the release of proteins sequestered in the mitochondrial inter-membrane space into the cytosol where they participate in caspases activation. In most cells, this event is considered as the no-return point of apoptosis. Therefore, understanding MOMP is one of the main stakes of research in this field (Borner, 2003; Cory et al., 2003).

Mitochondria are not static but dynamic organelles that continuously fuse and divide. It has been reported that mitochondrial morphology has an impact on apoptosis, although this remains controversial. In this review we will discuss how pro-apoptotic family members are thought to permeabilize mitochondria during

Abbreviations: MOMP, mitochondrial outer-membrane permeabilization; MOM, mitochondrial outer-membrane.

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apoptosis, we will also describe the mechanisms of mitochondrial dynamics and discuss how this process could participate in apoptosis.

2. Mitochondrial pathways of apoptosis

2.1. Two major pathways of apoptosis

Classically, two major pathways of apoptosis are distinguished. The extrinsic pathway that is engaged by ligands activating death receptors of the TNF/Fas family and the so-called intrinsic pathway, which involves mitochondria and is triggered by many stimuli, such as cytotoxic stress, DNA damage and growth factor deprivation. In some cells, cross-talk and cooperation between the two pathways have been described. We will focus on the proteins, mainly Bcl-2 family members, that integrate and convey the death signals to mitochondria, and on the events that occur at the level of mitochondria, i.e. fission of the organelle and permeabilization of the MOM.

2.2. The Bcl-2 protein family and its control of apoptosis

The BCL-2 gene (*B-cell lymphoma 2*) was originally identified as a proto-oncogene involved in the translocation t(14:18) of human follicular lymphoma (Tsujimoto et al., 1984). In contrast to many oncogenes, Bcl-2 does not trigger cell proliferation but promotes survival under deleterious growth conditions (Vaux et al., 1988). Bcl-2 is the prototype of a large family of proteins (more than 30 members), which share a high degree of homology although they exert different functions, being either anti-apoptotic or pro-apoptotic.

Anti-apoptotic proteins have usually four Bcl-2 homology (BH) domains (e.g. Bcl-2, Bcl-xL, Bcl-W and Mcl-1), whereas pro-apoptotic proteins display either three BH domains (BH1,2,3, e.g. Bax and Bak) or one only, the BH3 domain. The latter proteins are called BH3-only proteins (e.g. Bid, Bad, Bim, Noxa and Puma) and represent by far the most numerous proteins (Adams and Cory, 1998; Kroemer et al., 2007; Youle and Strasser, 2008).

The canonical three-dimensional structure of some Bcl-2 family proteins is a globular structure with 6–7 amphipathic α helices surrounding two central hydrophobic helices (Muchmore et al., 1996; Petros et al., 2001). This structure shares homologies with bacterial colicin and transmembrane domain of diphtheria toxin, two proteins able to form pores in membranes with their two hydrophobic α helices (Petros et al., 2004). Most Bcl-2 family proteins contain a C-terminal domain of about 20 hydrophobic residues, which can target them to the membrane of different compartments like mitochondria, endoplasmic reticulum or the nuclear envelope (Adams and Cory, 1998). Finally, the domains BH1, BH2 and BH3 of the Bcl-2 family form also a hydrophobic pocket (Suzuki et al., 2000). In the case of Bax, the hydrophobic N-terminal extremity of the protein can be folded into this hydrophobic groove, rendering the protein soluble in the cytosol (Nechushtan et al., 1999; Schinzel et al., 2004).

Many death stimuli appear to be integrated by the BH3-only proteins and then transduced from these proteins to other members of the family. According to crystallography studies, BH3-only proteins perform their activity by interacting with the hydrophobic groove of anti- or pro-apoptotic Bcl-2 members. Studies of protein–protein interactions allow to propose at least two models.

The "direct activation" model proposes that BH3-only proteins directly bind to Bax or Bak through their BH3 domain and recruit them into the MOM, where they can oligomerize and exert their permeabilizing effect. Not every BH3-only protein possesses this activity and so far only Bid, Bim, Map-1 and Puma have been reported to be direct activators (Cartron et al., 2004; Marani et al., 2002; Tan et al., 2001). It has recently been reported that a stabilized α-helix of Bim can stimulate Bax insertion by interacting at a site different from the canonical hydrophobic pocket, providing a new insight into the understanding of Bax activation (Gavathiotis et al., 2008). In the direct activation model, other BH3-only proteins could also act as inhibitor of anti-apoptotic members in order to free the BH3-only activators (Letai et al., 2002). The role of anti-apoptotic Bcl-2 proteins is to neutralize pro-apoptotic BH3-only proteins and thus inhibit their effect on Bax/Bak activity and MOMP.

In the "indirect" model, the role of BH3-only proteins is to bind to and neutralize anti-apoptotic members, such as Bcl-xL or Bcl-2. In this model, Bax and Bak are constitutively bound to their anti-apoptotic counterpart and released from their inhibitors during apoptosis. Consequently, Bax and Bak oligomerize and permeabilize the MOM without any further need of activation. Because not every pro-apoptotic protein are able to bind anti-apoptotic members, a combination of BH3-only proteins is required in this model for complete neutralization and MOMP. The role of anti-apoptotic proteins here is solely to inhibit Bax and Bak (Adams and Cory, 2007).

3. Bax/Bak-mediated mitochondrial outer-membrane permeabilization

In healthy cells, Bax is mainly located in the cytosol with a minor pool loosely attached to mitochondria (Hsu et al., 1997). Following certain apoptotic stimuli, BH3-only proteins like Bid and Bim can induce several structural changes in Bax structure. First its hydrophobic C-terminal part that was concealed within the hydrophobic pocket becomes exposed and targets Bax to the MOM. The N-terminal extremity of the protein becomes also exposed and is thus accessible to specific antibodies in mild-denaturing conditions (Lucken-Ardjomande and Martinou, 2005; Youle and Strasser, 2008). The protein undergoes a second conformational change that allows its α5 and α6 helices to insert into the MOM (Desagher et al., 1999; Hsu et al., 1997; Makin et al., 2001). Finally, Bax oligomerizes in the membrane and this results in MOMP and cytochrome c release (Antonsson et al., 2000; Eskes et al., 2000; Upton et al., 2007; Wei et al., 2000). Recently, Lovell et al. studied the steps of Bax activation by *Fröster Resonance Energy Transfer* (FRET) in liposomes (Lovell et al., 2008). They observed that Bid is rapidly targeted to membranes where it recruits Bax, inducing its insertion and oligomerization into the membrane (Fig. 1). The authors also reported that Bax activation can be prevented by Bcl-xL, which was shown to interact with Bid, but also with Bax, albeit with a lower affinity. Finally, addition of Bad was shown to neutralize Bcl-xL, thereby allowing the activation of Bax by Bid.

In contrast to Bax, Bak is constitutively inserted in the MOM where it is bound to and inhibited by VDAC2, Mcl-1 and Bcl-xL (Li et al., 2008; Willis et al., 2005). Similar to Bax, Bak oligomerization requires BH3-only proteins to induce MOMP (Wei et al., 2000). Early in Bak activation, the protein exposes its BH3-domain that interacts with the hydrophobic groove of another Bak molecule (Dewson et al., 2008). The resulting homo-dimers are thought to induce the nucleation of Bak and its complete activation.

Once activated, Bax and Bak induce MOMP, thus allowing the release of inter-membrane space proteins. The biophysics of the permeability transition step is not understood yet and has been extensively debated (Desagher and Martinou, 2000). Recent evidence suggests that Bax and Bak do not form a proteinaceous pore, but rather a lipidic pore devoid of protein molecules in its luminal part (Qian et al., 2008).

Finally, the dynamics of the mitochondrial network, particularly its fission during apoptosis, have recently been shown to influence mitochondrial outer-membrane permeabilization.

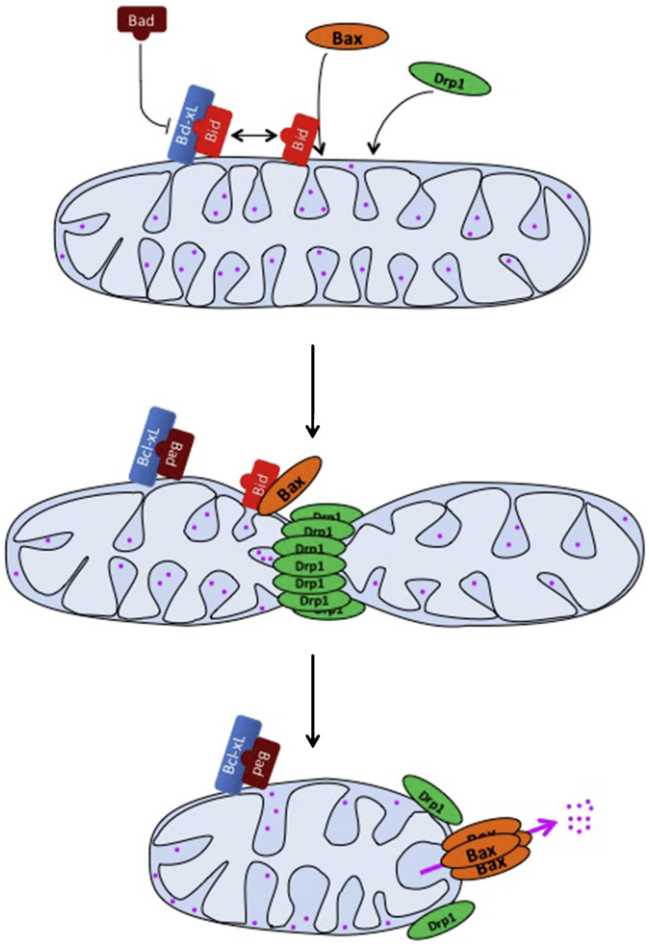

Fig. 1. Mitochondrial fragmentation during apoptosis. During apoptosis, Drp-1 is recruited to the mitochondria and forms a constriction ring around the organelle. At the same time, tBid inserts into the MOM and recruits Bax. Anti-apoptotic proteins (e.g. Bcl-xL), on the other hand, are inhibited by BH3-only proteins (e.g. Bad). In the MOM, Bax oligomerizes and permeabilizes the membrane by a mechanism that is still unclear. This results in the efflux of cytochrome c (pink dots) and many other proteins. A complete release of cytochrome c is allowed by the opening of the cristae junctions induced by the fission of mitochondria.

4. Mitochondrial dynamics

The mitochondrial network is fragmented concomitantly with MOMP (Arnoult et al., 2005a; Gao et al., 2001; Youle and Karbowski, 2005). In healthy cells, mitochondria cycle between several shapes and it is thought that their morphology results from the equilibrium between fusion and fission events. Because alteration in proteins that are responsible for mitochondrial morphology changes the sensitivity of cells to death, many laboratories have investigated the role of mitochondrial dynamics in apoptosis.

4.1. Mitochondrial fusion

The first gene found to be involved in mitochondrial dynamics, *Fuzzy Onion* (FZO), was identified in 1997 in *Drosophila melanogaster* (Hales and Fuller, 1997). FZO encodes a large GTPase of the MOM and is essential for spermatogenesis in the fly. The FZO protein has two paralogs in human: mitofusin 1 (Mfn-1) and mitofusin 2 (Mfn-2). Both mitofusins possess two transmembrane domains and are inserted into the MOM such that their N- and C-termini are in the cytosol (Rojo et al., 2002). Mitofusins contain also a GTPase domain that is essential for their activity, as well as two *heptad repeat* (HR) domains exposed to the cytosol, which allow the formation of *coiled coil* structures. Similar to SNAREs, trans-dimerization of mitofusins is responsible for mitochondrial tethering before fusion takes place although the process itself is insufficient to trigger membrane fusion (Detmer and Chan, 2007; Koshiba et al., 2004; Santel et al., 2003). In addition to their possibility of homo- and hetero-dimerization, mitofusins are regulated at the post-translational level (Suen et al., 2008).

The protein *Optic-Nerve Atrophy 1* (Opa-1) mediates mitochondrial fusion at the level of the mitochondrial inner-membrane (Cipolat et al., 2004; Olichon et al., 2002; Wong et al., 2003). Inner-membrane fusion is less understood but, like mitofusins, Opa-1 is a large membrane-bound GTPase that faces the inter-membrane space and is predicted to form *coiled coil* domains (Olichon et al., 2002). Opa-1 function is accomplished by its various isoforms. OPA-1 pre-mRNA can be differentially spliced and the protein can be further cleaved at up to two distinct sites resulting in many isoforms that are usually classified as “short” or “long” (Delettre et al., 2001). Opa-1 processing is mediated by several proteases of the mitochondrial inner-membrane such as the rhomboid protease PARL and the AAA metallo-proteases composed of YME1L1, SPG7, AFG3L1 and AFG3L2 (Cipolat et al., 2006; Delettre et al., 2001; Griparic et al., 2007; Ishihara et al., 2006; McQuibban et al., 2003; Song et al., 2007).

4.2. Mitochondrial fission

The principal actor of mitochondrial fission is the dynamin-related protein 1 (Drp-1) (Smirnova et al., 2001). Drp-1 is a protein of the dynamin’s superfamily that is present in the cytosol but also localizes at mitochondrial tips. Drp-1 oligomerizes around mitochondria and induces their fission in a GTP-dependent manner (Shaw and Nunnari, 2002; Smirnova et al., 2001). Drp-1 activity can be modulated by many post-translational modifications. In mitosis, Drp-1 is phosphorylated by Cdk1/cyclin B1, a modification that promotes its activity. Fission of mitochondria could allow a better distribution of the organelles between the daughter cells (Taguchi et al., 2007). Drp-1 can also be phosphorylated by cAMP-dependent protein kinase (or protein kinase A, PKA) which reduces its GTPase activity leading to the decrease of mitochondrial fission (Chang and Blackstone, 2007; Cribbs and Strack, 2007). Finally, the calmodulin-dependent kinase I α (CaM kinase Iα) can also phosphorylate Drp-1 following calcium influx (Han et al., 2008). Drp-1 phosphorylation by CaM kinase Iα results in an increased affinity of Drp-1 for mitochondria and induces mitochondrial fission. It remains to be clarified how Drp-1 phosphorylation on the same residue by PKA and CamKIα results in opposite effects. Drp-1 is actively dephosphorylated by calcineurin (PP2B) in a calcium-dependent manner, for example after mitochondrial depolarization (Cereghetti et al., 2008; Cribbs and Strack, 2007). Dephosphorylated Drp-1 accumulates at the surface of mitochondria where it fragments the network.

Drp-1 can be ubiquitinylated by MARCH-V (or MITOL), an E3-ubiquitin ligase residing on the MOM (Karbowski et al., 2007; Nakamura et al., 2006a; Yonashiro et al., 2006). However, whether MARCH-V induces mitochondrial fusion or fission is not clearly established yet because MARCH-V also interacts and regulates Mfn-2 and Fis-1 (Nakamura et al., 2006b; Yonashiro et al., 2006).

Finally, Drp-1 can also be sumoylated and protected from degradation by Sumo-1 (Harder et al., 2004). This post-translational modification induces its association with mitochondria while the sumo-ligase Sumo-1 is associated with the sites of mitochondrial fission. Interestingly, Drp-1 sumoylation is promoted by Bax and Bak during apoptosis (see later) (Wasiak et al., 2007). On the other hand, the sumo protease Senp-5 induces mitochondrial fission by

desumoylation and thus destabilization of Drp-1 (Zunino et al., 2007).

On mitochondria, Drp-1 interacts with Fis-1, a protein of the outer-membrane facing the cytosol (James et al., 2003; Yoon et al., 2003). Because Fis-1 over-expression induces a Drp-1-dependent mitochondrial fragmentation and Drp-1 and Fis-1 interact physically, it has been proposed that Fis-1 could be a receptor of Drp-1 on the MOM although loss of Fis-1 by RNA interference does not change the localization of Drp-1 and its assembly (Lee et al., 2004).

Additional proteins can influence mitochondrial fission but their mode of action is not clearly established yet. First, some Bcl-2 family proteins could modulate mitochondrial morphology as discussed in the next chapter. The modulator of Bax and Bak, Endophilin B1 (Bif-1) could also modulate mitochondrial morphology (Karbowski et al., 2004; Takahashi et al., 2005). In cells lacking this protein, fission of the inner membrane can occur without fission of the MOM. Another protein, Mtp-18, induces Drp-1-dependent mitochondrial fission (Tondera et al., 2004, 2005). Finally, over-expression of Gdap-1, a protein of the MOM, induces mitochondrial fission, whereas its loss of function induces mitochondrial elongation (Niemann et al., 2005; Pedrola et al., 2005).

5. Mitochondrial dynamics in apoptosis

5.1. The mitochondrial network is fragmented in apoptosis

Mitochondrial network fragmentation in apoptosis is dependent on Drp-1 as primarily shown by (Frank et al., 2001) (Fig. 1). Inhibition of Drp1 by RNA interference, use of a dominant-negative mutant of the protein (K38A) as well as a chemical inhibitor has been reported to delay cytochrome c release and cell death in many cell types (Cassidy-Stone et al., 2008; Estaquier and Arnoult, 2007; Parone et al., 2006). These data suggest that mitochondrial fission, which on its own is a physiological, non-deleterious process, can also participate in MOMP during apoptosis. Over-expression of Fis-1 has been shown to irreversibly fragment mitochondria and to promote cell death by inducing cytochrome c release (James et al., 2003). On the other hand, over-expression of Mfn-1 or Mfn-2 protects cells from MOMP (Sugioka et al., 2004). Over-expression of a constitutively active mutant of Mfn-2 exerts a cell protection more pronounced than that of wild-type Mfn-2, suggesting that the protein needs to be active to reduce the level of MOMP (Neuspiel et al., 2005). Similarly, over-expression of Opa-1 protects from apoptosis (Frezza et al., 2006). Together these data suggest that mitochondrial fission could participate in apoptosis. However, this process does not appear to be an absolute requirement for cell death (Estaquier and Arnoult, 2007; Parone et al., 2006; Sheridan et al., 2008).

The mechanisms by which Bax and Bak induce mitochondrial fission in apoptosis are not clear. Bax has been reported to colocalize with Drp1 at mitochondrial fission sites (Karbowski et al., 2002). Moreover, a recent study has shown that Bax and Bak induce Drp-1 sumoylation resulting in its stable association with mitochondria (Wasiak et al., 2007). Once the outer-mitochondrial membrane is permeabilized, DDP/Timm8a, an inter-membrane space protein that is released at the same time as cytochrome c can also activate Drp-1 in a retro-active pathway and promote the complete fragmentation of the mitochondrial network (Arnoult et al., 2005b). Finally, some soluble isoforms of Opa-1 are also released from the inter-membrane space after MOMP and have been proposed to induce fission of the mitochondrial network during apoptosis (Arnoult et al., 2005a). This would favour the hypothesis that fission of mitochondria occurs after MOMP.

5.2. OPA1 controls cristae morphology and cytochrome c availability for release

Inside mitochondria, cytochrome c is present in two different locations: a minor pool is free in the inter-membrane space, and a major pool enclosed in cristae (Delivani et al., 2006). Cytochrome c would be released in two successive steps after MOMP: first, the pool of cytochrome c that is soluble and then the pool present in cristae (Ott et al., 2002; Scorrano et al., 2002; Sun et al., 2007). It is possible that in some cell types the minor pool of cytochrome c is not sufficient for the formation of the apoptosome and the activation of caspases. Thus, the process of cristae opening would play an important role in apoptosis (Fig. 1).

Beyond its role in mitochondrial inner-membrane fusion, the large GTPase Opa-1 controls the morphology of cristae and their opening in apoptosis (Arnoult et al., 2005a; Cipolat et al., 2006; Frezza et al., 2006). Opa-1 over-expression decreases cytochrome c release and protects from apoptosis whereas its inhibition by RNA interference sensitizes cells to apoptosis and even induces spontonaneous cytochrome c release (Frezza et al., 2006; Olichon et al., 2003; Sugioka et al., 2004). Over-expression of Opa-1 in fusion-deficient cells (Mfn-1/Mfn-2 double knock-out for instance) protects also from apoptosis (Frezza et al., 2006). The anti-apoptotic effect of OPA-1 could be mediated by its ability to close cristae junctions and to sequester cytochrome c. Recently, a mutant of Opa-1 that is resistant to oligomer disassembly during apoptosis maintains cristae junctions closed and confers resistance to apoptosis (Yamaguchi et al., 2008). Short forms of Opa-1 could be responsible for closure of cristae junctions (Arnoult et al., 2005a; Frezza et al., 2006). OPA1 oligomer disassembly appears to depend on tBid but to be independent of Bax and Bak oligomerization and MOMP (Yamaguchi et al., 2008). The importance of cristae remodelling for cytochrome c release and apoptosis remains an open question (Reed and Green, 2002; Suen et al., 2008).

The mechanism by which Opa-1 modulates cristae junction opening is influenced by cleavage of this protein. The rhomboid protease PARL interacts with Opa-1 and is required for mitochondrial cristae integrity. Mitochondria deficient for PARL show disorganized cristae and are more sensitive to stress-induced or tBid-induced apoptosis (Cipolat et al., 2006). However, it is still unclear whether PARL can process directly Opa-1 and what is the involvment of AAA-proteases in this process.

5.3. A role of Bcl-2 family members in the regulation of mitochondrial morphology in healthy cells

The cooperation between Bcl-2 family members and proteins involved in mitochondrial dynamics during apoptosis raises the possibility that these proteins may also cooperate in healthy cells. Karbowski et al. reported that cells depleted of both Bax and Bak have fragmented mitochondria, which is not the case for single Bax or Bak depletion (Brooks and Dong, 2007; Karbowski et al., 2006). This suggests that Bak and Bax may exert a redundant function in the regulation of mitochondrial morphology. These observations were supported by the immunoprecipitation of Bax with Mfn-2 and Bak with Mfn-1 and Mfn-2 (Brooks et al., 2007). Bax binds Mfn-2 in its GTP-bound state, regulates its assembly in large complexes and changes its sub-mitochondrial distribution and membrane motility, resulting in a change in mitochondrial morphology. Finally, Bcl-2, Bcl-xL and the *Caenorhabditis elegans* homolog of Bcl-2 Ced-9 were also shown to interact with Mfn2 and regulate mitochondrial morphology (Delivani et al., 2006; Li et al., 2008). Bcl-xL has also been reported to regulate mitochondrial morphology and mitochondrial biomass in neurones by modulating fusion and fission (Berman et al., 2009). The possibility that some Bcl-2 family members could

regulate mitochondrial morphology is intriguing and requires further investigations.

6. Conclusion

Mitochondrial outer-membrane permeabilization is a key step in apoptosis and is under the control of Bcl-2 family proteins Bax and Bak. The precise mechanisms of activation of these proteins is not completely understood, despite recent considerable progress. It is even less understood how these proteins, once oligomerized, permeabilize the MOM. The role of mitochondrial fission in MOMP is still controversial, although it is clear that inhibiting mitochondrial fission clearly delays cytochrome c release. Whether this delay results from abnormal cristae junction opening or whether it is the consequence of an impact of mitochondrial fission on Bax and Bak oligomerization and MOMP remains an open question.

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