Mitochondrial Regulation of Apoptosis

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Mitochondria play a central part in cellular survival and apoptotic death. These processes are highly regulated by pro- and antiapoptotic Bcl-2 superfamily members. A key feature within apoptosis cascades is disruption of mitochondrial transmembrane potential and apoptogenic protein release, caused by opening of the permeability transition pore (PT). New data, however, indicate that mitochondrial apoptosis may occur without PT involvement.

Fundamental metabolic processes occur in mitochondria, which are essential for the survival of all eukaryotic cells. Apoptosis, however, is also controlled within these organelles. Examples of survival functions are the oxidative phosphorylation of adenine, the electron transport in the respiratory chain, the β-oxidation of fatty acids, as well as the citric acid cycle (Krebs cycle) and parts of the urea cycle. Regulation of apoptosis includes activation of certain membrane channels and release of apoptogenic proteins from the mitochondria into the cytosol, where they activate the terminal elements of a protease cascade pathway, which are capable of nuclear DNA fragmentation.

The architecture of the mitochondria generates two distinctive compartments that are exceptionally different in morphology and function. The outer mitochondrial membrane (OMM) is a common phospholipid bilayer with insertions of voltage-dependent anion channels (VDAC) permeable for solutes of up to 5 kDa. Small molecules such as energetic phosphates (ADP/ATP) or certain larger nonelectrolytes such as inulin may pass easily. Ion transition largely depends on their electric charge. In the open state, there is a high prevalence for anions. The inner mitochondrial membrane (IMM) is permeable only for water, oxygen, and carbon dioxide, thus generating two separated spaces: the intermembrane space and the mitochondrial matrix. The IMM is highly wrinkled to increase its surface for the metabolic activities described above and to guarantee brief distances between matrix and IMM. These wrinkles form lamellar structures, which are termed cristae. The IMM is the place where the key metabolic function, oxidative phosphorylation, is performed. Many apoptogenic proteins such as cytochrome c or the apoptosis-inducing factor (AIF) are sited here as well. About 90% of cytochrome c is stored in vesicles created by infoldings of the IMM; the remaining 10% is located in the intermembrane space. Cytochrome c physiologically functions as an electron transfer protein in many different redox processes.

In the mitochondrial matrix, enzymes for the Krebs cycle, water, oxygen, carbon dioxide, as well as recyclable intermediates and mitochondrial (mt) DNA are stored. Besides the reported functions, much attention has been recently drawn to inheritance of mtDNA, protein translocation, mitochondrial fusion, and activation and regulation of apoptotic cell death, which is discussed in this brief review.

The apoptosis cascades

Several dozens of apoptotic stimuli have been described over the last decade. Depending on the experimental settings as well as on the methods used for apoptosis detection, contradictory results about the signal transduction pathways can be found in the literature. However, it is a common belief that two main apoptosis cascades, activated by extrinsic or intrinsic stimuli, exist.

Although there is some overlap between these two pathways, cell-external stimuli such as cytokine release predominantly activate cell membrane-bound death receptors by specific ligation. These receptors belong to the TNF superfamily and are diverse in primary structure but have common cysteine-rich extracellular subdomains that adopt similar tertiary arrangements. Furthermore, they share a homologous intracellular sequence called a death domain, to which specific adapter molecules such as TNF receptor-associated death domain or Fas-associated death domain (FADD) can attach. The intracellular signal transduction to the nucleus is carried by caspases (cysteine aspartate-specific proteases). As of 2002, 14 caspases have been described, which were numbered in order of their discovery and not according to their hierarchical order. The complex of Fas/Fas ligand assembles to FADD via their death-effector domains. FADD recruits procaspase 8 to form a death-inducing signal complex (DISC). Procaspase 8 is autoactivated at DISC and becomes the active enzyme caspase 8, which is released into the cytosol. At the end of the caspase cascade activation is the cleavage of caspase 3, which may activate DNA fragmentation via the DNA fragmentation factor. Caspase 8, however, can also truncate cytoplasmic Bid into tBid, which then acts on the OMM by pore formation and release of proapoptotic proteins.

The mitochondrial regulation of apoptosis is usually aci-
vated by cell-internal stimuli such as reactive oxygen species, which can be formed by several different mechanisms, e.g., mild ischemia, as byproducts of cellular respiration, or ionizing radiation. This path is independent of the cell membrane receptor-mediated activation of apoptosis as described above (14). Recent papers, however, challenged this view of stress-induced mitochondrial-only apoptosis. Lassus and coworkers (6) demonstrated that stress-stimulated cytokines can directly activate caspase 2. In that scenario, mitochondrial permeabilization may function as amplification of caspase activation. The final steps of death signal transduction to the nucleus and subsequent DNA fragmentation by specific nucleases, however, are shared by both conduits, the receptor- and the mitochon-
drial-initiated apoptosis. Figure 1 illustrates the two principal pathways of apoptosis induction.

How mitochondrial apoptosis is regulated

Players in mitochondrial apoptosis. Regulators of mitochondrial apoptosis can be categorized according to their location. Apoptosis-relevant proteins of the OMM are the VDAC and Bcl-2 members. The adenine nucleoside translocator (ANT) is situated in the IMM. The mitochondrial creatine kinase (mtCK) resides between the VDAC and the ANT in the intermembrane space. The majority of apoptogenic proteins are stored in the intercristal space, whereas cyclophilin D and cardiolipin, both associated with the permeability transition pore (PT), are located in the mitochondrial matrix (see Fig. 2).

The role of VDAC in apoptosis. VDAC or porins are the major permeability pathways through the OMM. Transport through VDAC is bidirectional. An example of an inward-oriented process is the recently identified fast diffusion of Ca2+ from endoplasmic reticulum release sites to the IMM. Mitochondria with abundant expression of VDAC render these cells more susceptible to ceramide-induced cell death, thus proving the key role of mitochondrial Ca2+ uptake in apoptosis. The prototype of an outward-driven process is the endoplasmic supply of ATP, generated in the mitochondrial matrix. It is not immediately understandable why an ion channel with a size restriction of <5 kDa should function as a pore for apoptogenic proteins considerably larger than that. The clue lies in the conformational change of VDAC by proapoptotic members of the Bcl-2 family, namely Bim, Bak, and Bax (in association with Bid). For review, see Tsujimoto et al. (12).
**Bcl-2 superfamily members.** As of 2002, the mammal Bcl-2 superfamily consists of more than 20 members. The constituents are categorized according to their Bcl-2 homology domains (BH1–4) in the α-helical regions and according to their function in the apoptosis process (for review, see Ref. 2). Antiapoptotic members such as Bcl-xL, Bcl-w, or Bcl-2 itself are located in the OMM and inhibit apoptosis by preventing the opening of the VDAC in association with proapoptotic Bcl-2 members or by inhibition of the assembly of supramolecular openings formed by proapoptotic Bcl-2 members.

The proapoptotic Bcl-2 members are located primarily in the cytoplasm and can insert into the OMM on demand. These associates are categorized into members exhibiting BH1–3 domains (such as Bax and Bak) and into the BH3-only domain group (such as Bad or Bik; see Fig. 3). Pro- and antiapoptotic elements can form homo- and oligomers as well as heterodimers by binding to their BH domains. In fact, Bax monomers for example are ineffective in apoptosis induction, whereas tBid and Bim themselves are sufficient for pore formation by activation of the VDAC and subsequent release of apoptogenic substances into the cytoplasm. On the other hand, Korsmeyer and colleagues (4) recently reviewed published evidence that tBid can oligomerize Bax or Bak as well and that these complexes form large pores into the OMM through which cytochrome c is released. The main route of cytochrome c release in whole mitochondria, however, is probably through the PT, which is associated with matrix swelling and active expression of stored cytochrome c from the intercristal space. During apoptosis induction, the ANT conductance and permeability increase to 700 pS and 1.5 kDa, respectively. This leads to osmotic swelling of the mitochondrial matrix by water influx succeeded by compression of the intercristal space. The apoptogenic proteins are released from their intercristal storage into the intermembrane space. Some authors, however, reported cytochrome c release in the absence of IMM depolarization. The expelling of cytochrome c into the cytoplasm may be accomplished either by rupture of the OMM, which, however, is more likely in necrosis, or by escape through channels formed by Bax multimers with or without VDAC. Bax monomers do not exhibit apoptotic activity, and the antiapoptotic Bcl-2 itself inhibits VDAC channel formation. It is not yet clear whether caspases (2, 3, and 9) are really located inside mitochondria. The ejected proteins cytochrome c, caspases, ATP, and the cytoplasmic apoptosis protease activating factor-1 (APAF-1) associate in the cytosol to huge apoptosome complexes (700 kDa), which cleave downstream caspases. Alternative theories by Vander Heiden et al. (13) as well as by Matsuyama and Reed (7) are discussed in the text.

**FIGURE 2.** Mitochondrial regulation of apoptotic cell death. The main components of the permeability transition pore (PT) are the VDAC and the adenine nucleoside translocator (ANT), which are adjacent in the OMM and IMM, respectively. Besides these abundant proteins, peripheral benzodiazepine receptor (PBR) and Bax/Bcl-2 in the OMM and cyclophilin D and cardiolipin in the IMM complete the PT complex. Under normal conditions, the PT serves as sensor for mitochondrial matrix pH, matrix Ca\(^{2+}\), gate potential, redox state, and others. The regular VDAC is permeable to solutes of up to 1 kDa and functions with the help of mitochondrial creatine kinase (mtCK) as a shuttle of respiratory chain substrates such as ATP from the mitochondrial intermembranous space to the cytoplasm. The ANT on the other hand is impermeable, which is necessary to generate the electrochemical potential for oxidative phosphorylation. The big apoptogenic proteins such as cytochrome c (12 kDa), second mitochondria-derived activator of caspases/direct inhibitor of apoptosis (IAP)-binding protein with low pi (smac/Diablo; 100 kDa), AIF (57 kDa), and others are predominantly stored in vesicles created by infoldings of the IMM (intercristal space). During apoptosis induction, the ANT conductance and permeability increase to 700 pS and 1.5 kDa, respectively. This leads to osmotic swelling of the mitochondrial matrix by water influx succeeded by compression of the intercristal space. The apoptogenic proteins are released from their intercristal storage into the intermembrane space. Some authors, however, reported cytochrome c release in the absence of IMM depolarization. The expelling of cytochrome c into the cytoplasm may be accomplished either by rupture of the OMM, which, however, is more likely in necrosis, or by escape through channels formed by Bax multimers with or without VDAC. Bax monomers do not exhibit apoptotic activity, and the antiapoptotic Bcl-2 itself inhibits VDAC channel formation. It is not yet clear whether caspases (2, 3, and 9) are really located inside mitochondria. The ejected proteins cytochrome c, caspases, ATP, and the cytoplasmic apoptosis protease activating factor-1 (APAF-1) associate in the cytosol to huge apoptosome complexes (700 kDa), which cleave downstream caspases. Alternative theories by Vander Heiden et al. (13) as well as by Matsuyama and Reed (7) are discussed in the text.
but may increase its conductance and channel size up to 1.5 kDa on activation by other complexes of the PT. The channel opening leads to an influx of protons or small solutes followed by an osmotic water shift into the mitochondrial matrix.

Cardiolipin and cyclophilin D are located adjacent to the ANT in the mitochondrial matrix. Their functions under physiological conditions are manifold. In apoptosis regulation, both act as an opener of the PT. Whether this is a specific effect of the ANT remains questionable, since it has been shown in isolated OMMs (no ANT) that Bid induced Bax oligomerization and consecutive cytochrome c release was cardiolipin dependent.

The mtCK is bound to the outer leaflet of the IMM. In physiological states, this enzyme is vital to energy metabolism by catalyzing the transphosphorylation of creatine by ATP to phosphocreatinine and ADP. mtCKs form cubelike octamers in vivo that are crucial for the correct creatine phosphorylation and shuttle. In apoptotic cell death, the mtCK facilitates contact between the VDAC and ANT to form the PT megachannel at the contact sites of the mitochondrial membranes.

Apoptogenic proteins. At least seven different apoptogenic proteins, not counting the caspases, reside in the intermembrane space of the mitochondrion. Cytochrome c functions physiologically as carrier in the IMM, where it transfers electrons across the complexes of the respiratory chain. Cytochrome c is the best-described mitochondrial effector protein in apoptosis, leading to caspase activation on cytoplasmic release.

Similarly, AIF is a phylogenetically old protein of the oxidoreductase system of the IMM. The electron carriage is performed via its NADH and FAD binding domains. In apoptosis, AIF is released from the intermembrane space into the cytoplasm and further into the nucleus, where it provokes caspase-independent chromatin condensation and subsequently DNA fragmentation. Its function in the redox system can clearly be discerned from its ability of nuclear fragmentation during mitochondrial apoptosis induction. Deletion of large parts of the protein, including the NADH and FAD binding sites, inhibits the redox activity but not the nuclease activity, see Cande et al. (1).

Similar to AIF, endonuclease G, on mitochondrial release, does not cleave caspases but rather exhibits direct nuclease activity. Other apoptogenic proteins are heat shock protein 70; second mitochondria-derived activator of caspase (smac)/direct inhibitor of apoptosis-binding protein with low pl (Diablo); apoptosis protease activating factor-1 (APAF-1); caspases 2, 3, and 9; and HtrA2. It is not entirely clear whether caspases reside inside mitochondria (see Fig. 2).

Four theories about how the system may work

Many different theories of mitochondrial apoptosis induction and regulation have been proposed. Here we present those four models that we think are well supported by experimental data. The majority of experiments have been performed in synthetic membrane lipid bilayers with reconstitution of the VDAC under the influence of Bcl-2 members, but isolated mitochondria and cultured cells were also investigated. These different experimental setups may explain the diversity of observations. A cartoon of how it may work in vivo is illustrated in Fig. 2.

The PT-dependent pathways.

BAX/BAK AND VDAC FORM MEGACHANNELS. This model has been
introduced and propagated by Tsujimoto and Shimidzu (12). The authors propose that activation of mitochondrial apoptosis by an internal stimulation causes excessive Ca\(^{2+}\) influx and subsequently increased ANT conductivity. This is followed by an inward flux of protons and ions through ANT. The increasing matrix osmolality causes water attraction and mitochondrial swelling, apoptogenic protein release from the intercrystal storage, and escape into the cytosol by megachannels or ruptured OMM. The severe disruption of the mitochondrial membrane potential in necrosis, however, predominantly leads to extensive ATP loss and caspase-independent cell death.

**Bcl-xL may be necessary for physiological VDAC ATP transport.** Vander Heiden and coworkers (13) advanced a theory based on experimental data of OMMs from isolated mitochondria from growth hormone-depleted cells. Apoptosis might be induced by blockade of the normal VDAC function by proapoptotic Bcl-2 members. The authors proposed that, under normal conditions, proapoptotic Bcl-2 members could simply act as ion channels to dissipate a potential across the OMM. Bcl-xL would permit VDAC to remain open under stress and perform its main physiological function, which is ATP shuttling from the mitochondrial matrix into the cytoplasm. Closure of VDAC by Bax or Bak may lead to mitochondrial ATP accumulation, ANT malfunction, followed by the same steps as described above, mitochondrial swelling, and cytochrome c release.

**The PT-independent pathways.**

**Cytochrome c release without depolarization of the IMM.** Recently, Kuwana et al. (5) have shown in a cell-free system and with vesicular reconstitution of defined proteins that permeabilization of the OMM requires neither the mitochondrial matrix nor the IMM (5). BH3-domain peptides were sufficient for Bax oligomerization into the OMM. These oligomer pores in the OMM were so large that dextrans with a size of up to 2 mDa could pass through. This process could be blocked by Bcl-xL, an antiapoptotic member of the Bcl-2 family. Similarly, Finucane et al. (3) reported that permeabilization of the IMM might be secondary or subsequent to changes in the outer membrane, resulting from proteolytic degradation.

**PH AND ELECTRICAL POTENTIAL CHANGES ACROSS THE IMM INDUCE APOPTOSIS.** In normal mitochondria, protons are pumped across the IMM in conjunction with electron transport through the respiratory chain. Additionally, transporters such as the Na\(^+/\)K\(^+\) exchanger and the H\(^+\)/K\(^+\) antipporter regulate the pH gradient across the IMM. In contrast, H\(^+\) can escape through the OMM via the VDAC. PT activation can also be achieved by matrix alkalization and IMM hyperpolarization. Under some circumstances, pH changes could be observed before cytochrome c release and caspase activation. The pH influences the ability of Bcl-2 family members to form homo- and heterodimers. Furthermore, Bcl-2 channels created in planar lipid bilayers at neutral pH are voltage dependent. To generate a potential difference and opening of the Bcl-2 member channels, an ATPase/H\(^+\) pump is required. This pump drives ATP synthesis under physiological conditions via H\(^+\) back-leak from the intermembrane space into the matrix. It has been shown in yeast that Bax-resistant phenotypes exhibited a defect in this ATPase. Other possible effects of mitochondrial pH changes on apoptosis include the pH-dependent insertions of Bcl-2 members into the OMM. Cytosolic alkalization, for example, was correlated with Bax translocation to and insertion in the OMM. For review, see Motsumaya and Reed (7).

In summary, an activation of the PT is not a prerequisite for mitochondrial apoptosis, at least in reconstituted lipid bilayer channels. In these models, a permeabilization of the OMM, enough for cytochrome c release, can be induced without the PT. In fact, cytochrome c release may precede the permeability transition of the IMM. In vivo, however, the PT-induced mitochondrial apoptosis is probably the most common form.

**Discrete simulation of the apoptosis effector phase**

Experimental studies on complex processes such as mitochondrial apoptosis regulation are labor and time consuming, and kinetic details of metabolic pathways cannot be discerned well. Additionally, the results heavily depend on the experimental setup, and adjustment for many confounding variables is not possible. Furthermore, high-throughput techniques generate an enormous amount of data, which does not necessarily guarantee more information. Therefore, unified representations of cellular networks were designed to facilitate mitochondrial apoptosis modeling.

**Lattice molecular automata (LMA) is one example of the simulation methods (10).** The LMA is a cellular automata-based framework tailored toward the simulation of inter- and intracellular molecular network dynamics. Due to the conceptual simplicity of cellular automata, complex intracellular control mechanisms with a large number of interacting molecular species can be modeled to reveal the time-dependent dynamics of different intracellular pathways. Molecular entities, such as the members of the apoptosis cascade, are represented on a simulation grid, and the molecular dynamics are driven by homo- and heterodimerization events between members of the Bcl-2 family and respective docking of Bcl-2 members to mitochondrial membranes, thereby triggering further events of the apoptosis cascade.

We implemented an LMA for the discrete modeling of the highly complex mitochondrial regulation by Bcl-2 family members (11). Constant low-level expression of Bcl-2 family members resulted in an equilibrium situation, in which homo- and heterodimerization between the individual proteins prevented the coupling of free, proapoptotic members to the mitochondrial membrane. This balanced, stable situation prevented the opening of the permeability transition pore. If external stimuli were applied, however, increased expression of apoptosis promoters was detected, resulting in abundant cytochrome c release. The results of this simulation were a good fit for the common understanding of mitochondrial regulation of the apoptosis effector phase, and therefore they

**“Mitochondrial apoptosis is an evolutionary strictly conserved suicidal mechanism.”**
clearly complement experimental efforts.

Our opinion

Mitochondrial apoptosis is an evolutionary strictly conserved suicidal mechanism. The power of cellular self-destruction lies in its ability to be conducted in a variety of different ways. Further studies in isolated mitochondria and whole cells will unravel the continuing mystery of mitochondrial induction of apoptosis in vivo. We think that which pathway will be affected largely depends on the nature of the apoptotic stimuli.

We acknowledge the excellent artwork of Sabine Hauer.

Financial support was provided by Austrian Science Fund Grant no. P15679.

References