Hypothesis

Why are mitochondria involved in apoptosis?

Permeability transition pores and apoptosis as selective mechanisms to eliminate superoxide-producing mitochondria and cell

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Abstract Petit and co-authors have recently summarized results of their studies on the involvement of mitochondria in apoptosis [Petit et al. (1996) FEBS Lett. 396, 7–13]. The mechanism consists in the release to the cytosol of a protein (presumably a protease) that is normally sequestered in the intermembrane space of mitochondria. This protein, when added to isolated nuclei, caused typical apoptotic changes. Its release from mitochondria was shown to occur as a result of disruption of the outer mitochondrial membrane due to swelling of mitochondria caused by opening of so-called permeability transition pores in their inner membranes. Increase in the level of products of the one-electron reduction of O_2 (reactive oxygen species, ROS) is known to induce the mitochondrial pores. The hypothesis described here assumes that pore formation and apoptosis are involved in the organization of a defense system preventing ROS formation. It is proposed that ROS-induced pore opening lowers ROS production due to (a) maximal stimulation of mitochondrial O_2 consumption and, hence, intracellular $[O_2]$ lowering and (b) complete dissipation of mitochondrial membrane potentials and, as a consequence, maximal oxidation of such respiratory chain carriers as $CoQ^{\cdot-}$ which serve as one-electron O_2 reductants. ROS decrease allows pore closure. If, nevertheless, ROS are still accumulating in a mitochondrion, long-lived pores cause degradation of the organelle which cannot import and synthesize proteins due to the absence of the membrane potential. In this way, ROS-producing mitochondria can be eliminated (mitochondrial selection). Another result of the long-lived pores is mitochondrial swelling. This disrupts the outer mitochondrial membrane and releases the apoptosis-inducing protein. Apoptosis eliminates ROS-producing cells (cell selection).

Key words: Mitochondria; Apoptosis; Permeability transition pore; Reactive oxygen species; HIV

1. Mitochondrion-mediated apoptosis

According to recent observations by Kroemer, Petit and their colleagues [1–4], at least one of the mechanisms of programmed cell death (apoptosis) can be described as the following chain of events.

1. A stimulus causing apoptosis affects the cell in such a way that so-called permeability transition pores are opened in the inner mitochondrial membrane (step 1 in Fig. 1).
2. Since the pores are permeable for compounds of molecular mass < 1.5 kDa, which are normally responsible for osmotic balance between the mitochondrial matrix and the intermembrane space, this balance comes to depend on high-molecular-mass compounds impermeable for the pores. Their concentration is higher in the matrix than in the intermembrane space and cytosol. Therefore, water reaches the matrix, causing its swelling. As a result, mitochondrial cristae straighten and the outer membrane is broken since its area is smaller than that of the inner membrane (Fig. 1, step 2).
3. A special 'cell suicide protein' or 'apoptosis-inducing factor' (AIF), sequestered in the intermembrane space, is released into the cytosol. It reaches the nucleus and causes apoptotic changes there (Fig. 1, step 4).

Each of these steps was directly proved by Kroemer's and Petit's experiments and supported by many pieces of indirect evidence published by other groups (reviewed in [1]; see also [5,6]). The most important facts favoring the scheme described above are:

1. The pore opening is shown to be a very early event in apoptosis; factors preventing this opening prevent apoptosis;
2. The cell suicide protein found in the mitochondrial intermembrane space is released into the medium when pores are opened and swelling of matrix develops. The purified protein, when added to isolated nuclei, induces in them typical apoptotic changes.
3. Apoptogenic activity of the purified protein is blocked by *N*-benzylcarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD.fmk), already known as an inhibitor of the so-called interleukin-converting enzyme (ICE), i.e. the protease converting pro-interleukin-1β to interleukin-1β [5,7]. This fact is consistent with the observation that one of the proteins involved in apoptosis in the nematode *Caenorhabditis elegans* has a similar sequence and properties of the pro-interleukin-1β-processing protease [5,7].
4. z-VAD.fmk blocks apoptosis when added to intact cells (mammalian and insect cells have been studied).

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Abbreviations: ROS, reactive oxygen species; TNP, tumor necrosis factor; z-VAD.fmk, *N*-benzylcarbonyl-Val-Ala-Asp-fluoromethylketone

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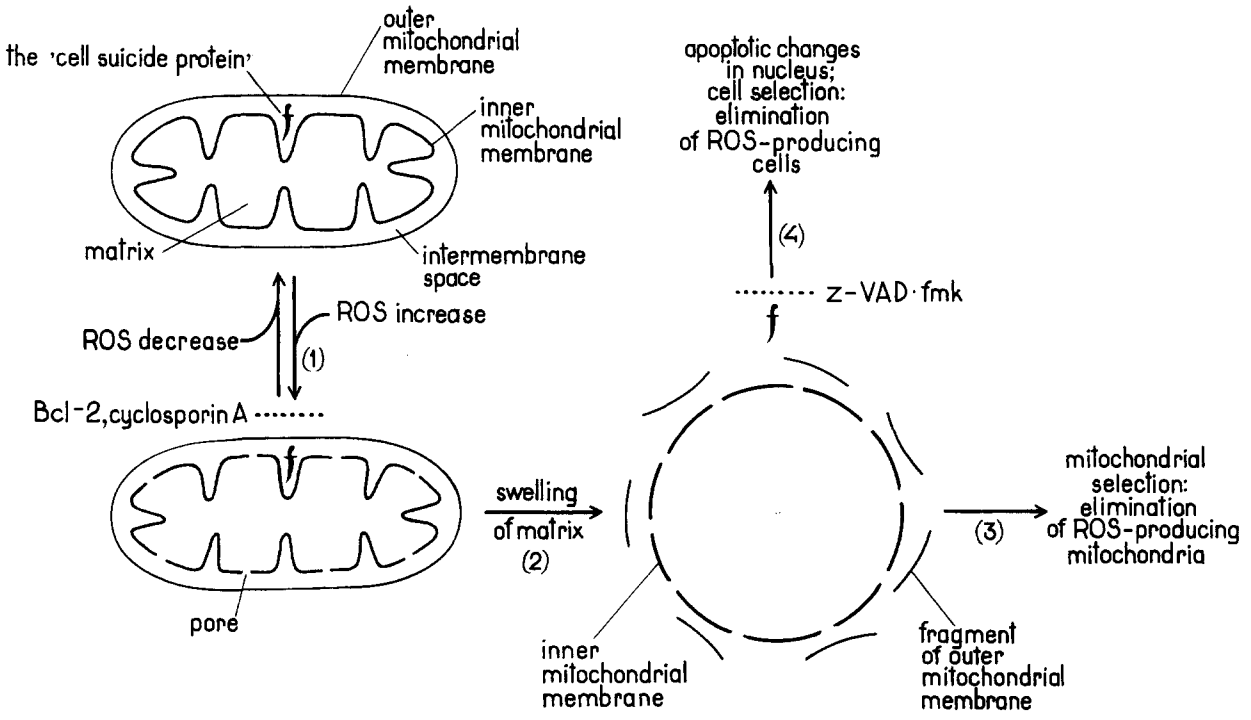

Fig. 1. Reactive oxygen species, mitochondria, and apoptosis. One-electron reduction of O_2 (producing $O_2^{\cdot-}$ and other ROS) and some other apoptosis-initiating factors entail opening of pores in the inner mitochondrial membrane (step 1). This stimulates O_2 consumption by mitochondria, decreasing the concentration of intracellular [O_2] and one-electron O_2 reductants and, hence, ROS production. [ROS] decrease allows pore closing. If, however, ROS are still accumulating, long-lived pore opening causes an osmotic disbalance between the mitochondrial matrix and the intermembrane space and swelling of the matrix. As a result, the mitochondrial cristae straighten and the outer membrane is broken since its area is smaller than that of the inner membrane (step 2). Long-lived pores prevent import to mitochondria of protein precursors encoded in the nucleus since this process requires membrane potential which collapses when pores are opened. This results in degradation of those mitochondria that form ROS in spite of the pore opening (step 3). The proteins sequestered in the intermembrane space are released into the cytosol. Among them is a protein that causes apoptotic changes in the nucleus (the 'cell suicide protein'). When a sufficient number of mitochondria become swollen, the concentration of this protein in the cytosol reaches a threshold, and apoptosis is initiated. In this way ROS-produced cells are eliminated (step 4). Inhibitors of pore opening (cyclosporin A, Bcl-2) and the inhibitor of the cell suicide protein z-VAD.fmk interrupt this chain of events at its beginning and at its end, respectively.

2. Some questions to be answered

The main question arising when we consider the above scheme is why do cells employ such a complicated and, on the face of it, even illogical mechanism? The following problems should be considered:

1. Why is the cell suicide protein hidden in the intermembrane space of mitochondria, organelles responsible for respiration and aerobic energy supply of the cell?
2. Why is such a multistage process of its release employed (pore opening → swelling of matrix → outer membrane break-down) although mitochondria already possess a delicate mechanism of protein translocation across the outer mitochondrial membrane which is used, e.g. to transport apocytochrome *c* [8]?
3. Why does uncoupling of oxidative phosphorylation stimulate pore formation which results in the complete inhibition of aerobic ATP synthesis when pores are opened? In this context, it should be stressed that apoptosis appears a well-organized process which certainly requires energy. On the other hand, the pore opening converts the mitochondrion from a cellular 'power station' supplying the cell with utilizable energy, to a 'furnace' consuming oxygen and burning nutrients without any energy conservation.

To answer these questions, let us consider the assumption that during evolution the primary function of apoptosis was (and perhaps now remains) the elimination of cells that become producers of large amounts of reactive oxygen species (ROS).

3. Uncoupling, pore opening, and apoptosis as three lines of anti-ROS defense

In 1994, I hypothesized [9] that aerobic cells have developed a special protective system composed of several lines of defense against oxygen toxicity, *preventing* ROS formation (see [6,10] for details). This system was assumed to operate in parallel with the well-known antioxidant mechanisms detoxifying *already formed* ROS. In animal cells, the ROS formation-preventing system might be organized as follows.

1. 'Mild' uncoupling of respiration and phosphorylation by means of an increase in proton leak through the inner mitochondrial membrane. This effect is assumed to appear under the activity-to-rest transition when the main mechanism of oxygen consumption (i.e. phosphorylating respiration) is switched off due to the exhaustion of ADP and the accumulation of ATP. When ADP is exhausted, intracellular O_2 concentration increases because of the inhibition of respiration. Simultaneously, the life-

time of $CoQ^{\bullet -}$ becomes much longer due to inhibition of the Q-cycle. $CoQ^{\bullet -}$ is an excellent one-electron O_2 reductant which can initiate ROS formation. Partial uncoupling stimulates O_2 consumption, lowers $[O_2]$, shortens the $CoQ^{\bullet -}$ lifetime, and as a result inhibits ROS production.

2. If mild uncoupling is insufficient to stop ROS formation, pores are opened. This results in the complete uncoupling of respiratory phosphorylation and leads to the maximal rate of respiration since respiratory substrates can now easily reach their intramitochondrial dehydrogenases. The resulting rapid oxygen consumption decreases $[O_2]$. The $CoQ^{\bullet -}$ lifetime is also minimized. It has been shown that the pore opening is stimulated by [ROS] increase, whereas [ROS] decrease allows pore closing [6,11]. If ROS still accumulate in spite of the appearance of pores, the affected mitochondrion will degrade since both the import of proteins to mitochondria [8] and synthesis of proteins in these organelles [12–14] require the membrane potential which cannot be maintained while pores are open. In other words, the pores can well be a mechanism for eliminating the ROS-producing mitochondria (Fig. 1, stage 3). In line with this logic, it was found that the development of apoptosis correlates with inhibition (a) of maturation of mitochondrial protein precursors encoded in the nucleus [15] and (b) of mitochondrial translation [16].
3. Long-lived pores cause swelling of mitochondria and, as a consequence, the disruption of the outer mitochondrial membrane. Thus, the cell suicide protein is released. If the number of such mitochondria increases, the concentration of the cell suicide protein in the cytosol reaches some threshold value required to initiate the apoptotic changes in the nucleus. As a result, ROS-producing cells are eliminated.

In fact, recent data reported by Kroemer et al. [1–4] confirm part of our hypothesis where pore formation is assumed to be a step toward apoptosis. It is noteworthy that an uncoupler and a prooxidant were shown to stimulate release of the cell suicide protein. The results of experiments on cells lacking mitochondrial DNA (ρ^0) seem to be the most interesting in this context. In these cells, apoptosis could be caused by tumor necrosis factor (TNF), but not by antimycin A. In the control cell line (ρ^+) which contains mitochondrial DNA, both agents were competent in inducing apoptosis [4].

The primary apoptosis-inducing signals are quite different in the cases of TNF and antimycin A. For a cell, TNF is a 'bad message', i.e. an order to commit suicide coming from the outside. On the other hand, antimycin A is a specific inhibitor blocking electron transport in cytochrome *b* immediately after heme b_h. If there is no cytochrome *b*, the antimycin A target is absent. Cytochrome *b* is encoded by mitochondrial DNA [17], and therefore is absent in the ρ^0 cell line.

The mechanism by which antimycin A initiates apoptosis in ρ^+ cells most probably consists of the stimulation of ROS formation, since inhibition of the respiratory chain after b_h greatly increases the lifetime of $CoQ^{\bullet -}$ [6]. Antimycin A-induced ROS accumulation has been known since the work of Boveris and Chance [18]. These authors also showed that uncoupling as an anti-ROS defense mechanism is inefficient in antimycin A-treated mitochondria. Even worse, uncoupler stimulates ROS formation when added after antimycin A [18].

The proposed hypothesis seems to answer questions concerning the complexity of the apoptotic mechanism.

1. The cell suicide protein is sequestered in mitochondria since (i) these organelles are the main source of ROS in the cell and (ii) apoptosis is employed as a mechanism discarding ROS-producing cells.
2. Involvement of uncoupling and pore opening in the apoptotic process is quite reasonable if we assume that these two events are the first two lines of anti-ROS defense, whereas apoptosis represents the last line.
3. Significant energy deficiency cannot occur at least during early stages of apoptosis if swelling of a rather small portion of the mitochondrial population in the cell is sufficient to reach the threshold concentration of the cell suicide protein required to initiate apoptotic modification in the nucleus.

It should be stressed that any agent causing pore opening induces mitochondrial swelling and the release of the cell suicide protein. This is why apoptosis, which is employed by the organism not only to prevent ROS formation but also for other purposes, can be initiated not only by ROS, but also by the other pore inducers. The known set of these inducers already includes some hormones, in particular, thyroxine [19].

4. Some perspectives

The hypothesis formulated in the paper allows several concrete predictions to be made.

1. Inhibition of the pore opening by, say, cyclosporin A or inhibition of the cell suicide protein by z-VAD.fmk (Fig. 1, steps 1 and 4, respectively) should increase ROS formation by cell populations.
2. z-VAD.fmk, preventing discharge of the ROS-producing cells, should increase the number of cells with swollen mitochondria. This may decrease the number of mitochondria per cell since pores prevent protein import and synthesis by mitochondria.
3. Injury in the first line of the anti-ROS defense, i.e. in the mild uncoupling mechanism, should be unfavorable for the maintenance of low ROS levels both in cells and in isolated mitochondria. In this respect, the search for natural mild uncouplers is very interesting. Thyroid hormones seem to be good candidates for this role [10,20].

One more intriguing problem is the relationship between ROS-linked apoptosis and apoptosis involved in systems other than the anti-ROS defense, such as elimination of virus-infected cells, ontogenesis, immune response, etc.

Preliminary analysis of the present situation in the problem 'mitochondrion and apoptosis' indicates that we are dealing here with a principle of general importance at least for the animal kingdom. The cell suicide protein was found by Kroemer and co-authors in cells of different tissues (hepatocytes, myelomonocytes, lymphoid cells) from two different species (mouse and man). Something similar is apparently present in *C. elegans* (see above). Infection of T-lymphocytes with HIV was shown to induce apoptosis [21,22] which is asso-

ciated with accumulation of HIV RNA in mitochondria which begin to swell [23].

Thus, the minimal hypothesis suggests that there is only one mechanism for realization of the death sentence for the cell (Fig. 1), just as the samurai always committed hara-kiri in the same manner.

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