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Minireview

Pro-oxidants and mitochondrial Ca^{2+}: their relationship to apoptosis and oncogenesis

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Apoptosis is a physiological process for active cell removal. One of its hallmarks is an increased cytosolic Ca^{2+} content. Several genes involved in apoptosis control have been identified, but their mode of action is not understood in detail. Apoptosis may relate to oncogenesis, in that some malignant tumors may grow because genes engaged in apoptosis control are altered. L929 cells overexpressing the proto-oncogen *bcl-2* have an increased mitochondrial membrane potential ($\Delta\psi$), as have many carcinoma cells. *bcl-2* protects L929 cells against apoptosis caused by pro-oxidant-induced mitochondrial Ca^{2+} 'cycling' and increased cytosolic Ca^{2+} levels. Nerve growth factor, which induces catalase, and inhibitors of mitochondrial Ca^{2+} release also prevent apoptosis. It is suggested that a pro-oxidant-induced Ca^{2+} release from mitochondria, followed by Ca^{2+} cycling and ATP depletion, is a common basic event during apoptosis. Accordingly, maintenance of $\Delta\psi$ stabilizes mitochondria, thereby prevents apoptosis, and may confer increased growth potential to cells.

$\Delta\psi$; *bcl-2* Proto-oncogen; Tumor necrosis factor; Nerve growth factor; Catalase

1. CELLULAR CA^{2+} HOMEOSTASIS

Free calcium ions play a prominent role in the regulation of many enzyme systems and serve the fundamental function of an intracellular messenger [1]. To this end, fine tuning of the Ca^{2+} concentration is required, which is achieved by binding of Ca^{2+} to non-membraneous proteins, and by membrane-bound Ca^{2+} transport systems. The latter carry the main burden of Ca^{2+} regulation. They operate in the plasma membrane, the endoplasmic (sarcoplasmic) reticulum, in the nucleus, and in mitochondria, and use, with the exception of the mitochondrial transport system, ATP hydrolysis as the driving force for Ca^{2+} transport. In light of the importance of the Ca^{2+} homeostasis it is not surprising that its disturbance can be fatal for the cell (reviewed in [2]).

2. MITOCHONDRIAL CA^{2+} TRANSPORT

Mitochondrial Ca^{2+} uptake occurs via a specific pathway and is driven by the mitochondrial membrane potential ($\Delta\psi$) (for review, see [3]). Intramitochondrially Ca^{2+} regulates three dehydrogenases and possibly nucleic acid synthesis. In addition, mitochondria are a safety device against a flooding of the cytosol with Ca^{2+}, because they can take up and store transiently large amounts of Ca^{2+} with impunity.

In order to prevent dysregulation of mitochondrial enzymes and calcification of the organelle, mitochondria must be able to release Ca^{2+}. Release from intact mitochondria occurs via a specific route in exchange with Na^+ or H^+, but in de-energized mitochondria release may also occur by reversal of the uptake pathway. Under normal conditions mitochondria slowly take up and release ('cycle') Ca^{2+}, and do so with little energy expenditure. However, under certain conditions, e.g. with high Ca^{2+} loads and oxidative stress, Ca^{2+} cycling becomes excessive and results in an increased energy demand and a dramatic fall of $\Delta\psi$. This leads to a decreased ability of mitochondria to retain Ca^{2+}, their uncoupling, and an impairment of mitochondrial ATP synthesis, which in turn deprives the cell of the fuel for the Ca^{2+}-sequestering ATPases. As a consequence, the cytosolic Ca^{2+} content rises drastically.

3. PRO-OXIDANTS CAUSE CA^{2+} RELEASE FROM MITOCHONDRIA

Pro-oxidants such as hydrogen peroxide or organic peroxides stimulate the Na^+-independent Ca^{2+} release

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Abbreviations: $\Delta\psi$, electrical potential across the inner mitochondrial membrane, negative inside; TNF, tumor necrosis factor; NGF, nerve growth factor.

*No distinction is made here between apoptosis, programmed cell death, and physiological cell death (see, for example, [18,58,59] for discussion).

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from intact mitochondria, as shown in isolated organelles and in cells (for review, see [4]). This release requires pyridine nucleotide oxidation followed by hydrolysis of NAD^+ to ADPribose and nicotinamide, and is inhibited by agents which prevent NAD^+ hydrolysis or protein mono(ADPribosyl)ation, e.g. by cyclosporin A or *m*-iodobenzylguanidine. These agents protect isolated mitochondria [5–7], cells [8–10], and organs [11] against Ca^{2+}-induced damage.

4. APOPTOSIS, ONCOGENESIS, PRO-OXIDANTS AND CA^{2+}

Apoptosis (programmed cell death, physiological cell death)* is a fundamental process observed during organ and body sculpting and control of immune cells [12–16], but may possibly be a general property of most cells [17,18]. Cells go into apoptosis as a response to a variety of primary triggers such as appearance or disappearance of hormonal signals, changes in cytokine and growth factor levels, or increased cytosolic Ca^{2+} levels. Apoptosis is often, but not always, accompanied by an activation of (a) Ca^{2+}-dependent endonuclease(s) and a characteristic fragmentation of nuclear DNA.

Apoptosis requires an active participation of cellular components, and several cellular genes (*bcl-2, c-myc, p53, TRPM-2, RP-2, RP-8, APO-1/FAS, ced-3, ced-4, ced-9*) have been identified which can control (suppress or stimulate) it. For example, the proto-oncogen *bcl-2* suppresses apoptosis induced by growth factor deprivation or viruses [19–23]. The mechanisms by which such a control is achieved are not clear. An important first hint comes from the recent report [24] that L929 cells overexpressing *bcl-2*, which is mainly localized in the inner mitochondrial membrane [25] but also in the nuclear envelope and endoplasmic reticulum [26,27], have an increased $\Delta\psi$ and are therefore protected against apoptotic cell killing (see below). In the present context it is important to note that mitochondria of many carcinoma cells have an increased $\Delta\psi$ [28]. Also viral genes can protect cells from going into apoptosis [29–31] or stimulate it [23].

Apoptosis was in the past mainly seen as a means to remove cells. However, it is now realized that inhibition of apoptosis may contribute to oncogenesis [32,33], because tissue homeostasis is a fragile balance between cell proliferation and cell death.

The importance of Ca^{2+} in cell proliferation has already been recognized many years ago [34,35]. For instance, oncogenic growth factors such as epidermal or platelet-derived growth factor cause an increase of the cytosolic Ca^{2+} level by stimulating Ca^{2+} influx into the cell or by release of Ca^{2+} from the endoplasmic reticulum through turnover of phosphatidyl inositols.

Pro-oxidants such as H_2O_2 and organic peroxides may play an important role in cell proliferation (for reviews see [36,37]). Depending on the level of exposure, pro-oxidants can both stimulate or inhibit proliferation of fibroblasts [38]. Pro-oxidants may act through mobilization of Ca^{2+}, as demonstrated in mouse epidermal cells [39]. In addition, protein kinases, especially protein kinase C [40] and the insulin receptor tyrosine kinase [41,42], can be activated during exposure of cells to oxidative stress. Hence, both an increase in the cytosolic Ca^{2+} concentration and an activation of protein kinase C may be critically involved in pro-oxidant-mediated cell proliferation. It is also worth noting that extracellularly generated oxygen radicals cause an increase in the rate of transcription of c-*myc* and c-*fos* genes [43], and that their expression is inducible by Ca^{2+} [44] as well as protein kinase C [45].

5. THE ROLE OF MITOCHONDRIAL CA^{2+}

Tumor necrosis factor α (TNF) induces apoptosis in L929 cells by stimulating superoxide radical production in mitochondria [46,47]. Resistant L929 cells, produced by culturing wild type cells in media containing increasing TNF concentrations, have a diminished ability to produce superoxide in response to TNF [47]. TNF-induced superoxide production and cell killing require mitochondrial Ca^{2+} cycling because they are suppressed by omission of Ca^{2+} or in the presence of EGTA, and by Ruthenium red, an inhibitor of the mitochondrial Ca^{2+} uptake pathway [47]. Increased mitochondrial superoxide production is followed by a decrease in mitochondrial functions as illustrated by the fall of dehydrogenase activity and ATP depletion [47]. L929 cells overexpressing the proto-oncogen *bcl-2* have mitochondria with elevated $\Delta\psi$, and are much less sensitve to TNF [24]. Indeed, nigericin, an ionophore which raises $\Delta\psi$, protects wild type cells even at high TNF concentrations [24]. These results suggest that TNF mediates apoptosis in L929 cells by stimulating mitochondrial Ca^{2+} cycling, and that *bcl-2* protects against it. *bcl-2* also blocks Ca^{2+}-triggered death in thymocytes [48,49] and cultured pancreatic cells (J.-C. Martinou, personal communication), and prevents apoptosis when expressed in *Caenorhabditis elegans* [50]. However, it should also be mentioned that *bcl-2* protects against apoptosis, induced by the protein kinase inhibitor staurosporine or by serum deprivation, in cells lacking mitochondrial DNA and therefore mitochondrial respiration [51].

Neurons require the presence of nerve growth factor (NGF) to remain viable in cell culture, but neurons overexpressing *bcl-2* do not need NGF to survive [52]. NGF protects neurons in vitro against perturbations of the pro-oxidant–antioxidant balance by the induction of catalase and glutathione transferase [53], and catalase enables resting chondrocytes to survive when grown in the absence of growth factors in serum-free culture [54].

Pro-oxidants like *tert*-butyl hydroperoxide, cumene hydroperoxide, or 3,5-dimethyl-*N*-acetyl-*p*-benzoqui-

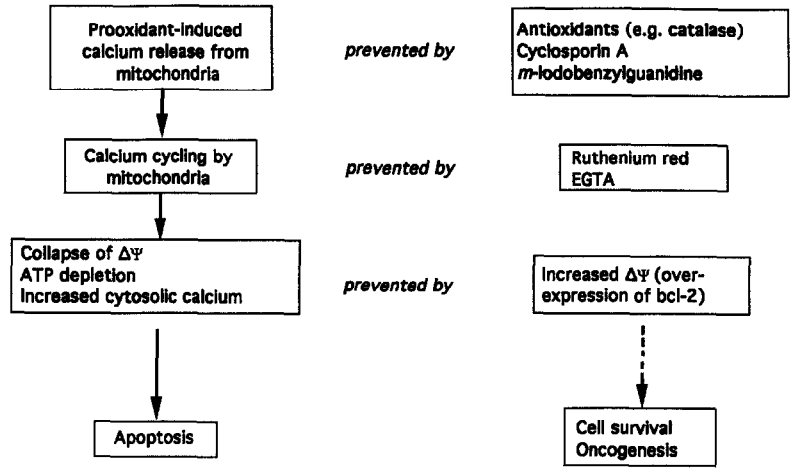

Fig. 1. Pro-oxidants and mitochondrial Ca^{2+} in apoptosis and oncogenesis. Pro-oxidants induce Ca^{2+} release from mitochondria, which may be followed by Ca^{2+} 'cycling', collapse of $\Delta\psi$, and ATP depletion. Under these conditions the cellular Ca^{2+} homeostasis cannot be maintained, the cytosolic Ca^{2+} level rises, and cells go into apoptosis. The pro-oxidant-induced Ca^{2+} release is prevented by antioxidants (e.g. catalase), or by compounds which prevent protein mono(ADPribosyl)ation in mitochondria (e.g. cyclosporin A or *m*-iodobenzylguanidine). Ca^{2+} re-uptake is prevented by Ruthenium red or Ca^{2+} chelators. The protein encoded for by the proto-oncogen *bcl-2* stabilizes $\Delta\psi$, thereby limiting ATP depletion and the disturbance of the cellular Ca^{2+} homeostasis. Inhibition of apoptosis (e.g. by overexpression of *bcl-2*) may confer increased survival potential to cells and thereby favour oncogenesis. $\Delta\psi$, electrical potential across the inner mitochondrial membrane, negative inside.

none imine deplete hepatocytes of ATP and kill them [8]. Cyclosporin A, a potent inhibitor of the pro-oxidant-induced Ca^{2+} release in isolated mitochondria [6,7], blocks mitochondrial Ca^{2+} release and therefore excessive Ca^{2+} cycling also in hepatocytes. Cyclosporin A thereby prevents loss of ATP, and keeps the hepatocytes viable [8]. Similar results were reported in [55]. Also infection of cells by viruses can result in mitochondrial Ca^{2+} cycling followed by cell death [56]. Whether it occurred by apoptosis was not tested in these experiments. However, apoptosis is inhibited by cyclosporin A in animals, as recently shown by analysis of hormone-induced regression of bullfrog tadpole tails [57].

6. HYPOTHESIS

Taken together, these results lead to the hypothesis that apoptosis can be triggered by a pro-oxidant-induced Ca^{2+} release from mitochondria, as summarized in Fig. 1. Ca^{2+} release causes a direct stimulation of the Ca^{2+}-dependent endonuclease(s), and/or a massive disturbance of the cellular Ca^{2+} homeostasis due to mitochondrial Ca^{2+} cycling, ATP depletion, and inhibition of membrane-bound ATPases, which normally remove Ca^{2+} from the cytosol. Accordingly, *bcl-2* (and maybe other proto-oncogens?) prevents cells from going into apoptosis by counteracting the collapse of $\Delta\psi$ and its fatal consequences. The proposed sequence of events also explains the need for NGF for growth of neurons in cell culture because NGF provides catalase and thereby lowers the level of hydrogen peroxide, a stimulator of mitochondrial Ca^{2+} release. In addition, the hypothesis suggests a causal link between the increased $\Delta\psi$ and the unlimited growth potential of many carcinoma and possibly other cancer cells.

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