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MINI REVIEW

Alteration of mitochondrial function and cell sensitization to death

Vladimir Gogvadze · Boris Zhivotovsky

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Abstract Stimulation of cell death is a powerful instrument in the organism's struggle with cancer. Apoptosis represents one mode of cell death. However, in a variety of tumor cells proapoptotic mechanisms are downregulated, or not properly activated, whereas antiapoptotic mechanisms are upregulated. Mitochondria are known as key players in the regulation of apoptotic pathways. Specifically, permeabilization of the mitochondrial outer membrane and subsequent release of proapoptotic proteins from the intermembrane space are viewed as decisive events in the initiation and/or execution of apoptosis. Disruption of mitochondrial functions by anticancer drugs, which induce oxidative stress, inhibit mitochondrial respiration, or uncouple oxidative phosphorylation, can sensitize mitochondria in these cells and facilitate outer membrane permeabilization.

Keywords Mitochondria · Apoptosis · Permeability transition · Cytochrome *c* · Cardiolipin

Introduction

In 1929 Warburg found that the glycolytic pathway accounts for much of the ATP production in cancer cells, even under aerobic conditions when, according to Pasteur, glycolysis would be suppressed. This phenomenon is now known as the "Warburg effect" (Warburg, 1956). Since then a high glycolytic rate, also in the presence of oxygen, has been known as a characteristic feature of cancer cells. Warburg originally suggested that aerobic glycolysis in cancer cells might reflect defects in mitochondrial energy production. He hypothesized that inhibition of mitochondrial function (for example by ionizing radiation) would reduce the activity of these organelles below a threshold level essential for cell survival, whereas mitochondria in normal cells would still be able to produce ATP. However, further studies revealed that tumor mitochondria are fully functional with regards to respiration and ATP synthesis (Nakashima et al., 1984). The present view on this problem associates the Warburg effect with alterations in signaling pathways that govern glucose uptake and its further metabolism rather than with mitochondrial defects. Once cells are autonomous with regards to their ability to take up nutrients, they are no longer dependent on the extracellular environment. This is important for cancer cell survival under conditions of rapid growth when oxygen supply is limited.

The increased dependence of cancer cells on glycolysis for ATP generation provides a biochemical basis for the design of therapeutic strategies to selectively kill cancer cells by pharmacological inhibition of the glycolytic pathway (Pelicano et al., 2006). It should be mentioned, however, that mitochondrial impact in cellular physiology is not restricted to ATP production for cellular demands. Mitochondria actively participate in the regulation of intracellular Ca^{2+} homeostasis, production of reactive oxygen species (ROS) which might be harmful if produced excessive, and are critically involved in the regulation of cell death pathways. Therefore, stimulation of processes that perturb mitochondrial stability and compromise their integrity might contribute to the elimination of cancer cells.

Apoptosis and cancer – opposing processes

Hanahan and Weinberg (2000) described six essential alterations in cell physiology that collectively dictate malignant

V. Gogvadze (✉) · B. Zhivotovsky
Institute of Environmental Medicine, Division of Toxicology, Karolinska Institutet, Box 210, SE-171 77 Stockholm, Sweden
e-mail: Vladimir.Gogvadze@ki.se

growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis and evasion of programmed cell death (apoptosis). Most of the anticancer drugs act at least in part through induction of apoptosis. Despite their relative resistance to apoptosis, tumor cells still contain the components of cell death machinery and can be sensitized to chemo- and/or radiation therapy by modulation of death signaling pathways (Viktorsson et al., 2005).

Although several modes of cell death are known, e.g. apoptosis, necrosis, autophagy and mitotic catastrophe, the molecular mechanisms of apoptotic cell death are so far best characterized. Apoptosis is an evolutionarily conserved and genetically regulated process of critical importance for embryonic development and maintenance of tissue homeostasis in the adult organism.

Oxidative stress and apoptosis

Apoptosis can be triggered by a variety of signals and pathophysiological conditions, including oxidative stress (Hampton and Orrenius, 1998). ROS are implicated in numerous pathologies, including malignant diseases, type II diabetes, atherosclerosis, chronic inflammatory processes, ischemia/reperfusion injury, and several neurodegenerative diseases (Droge, 2002). In addition, ROS play a regulatory role in cellular metabolic processes by activation of various enzymatic cascades and transcription factors.

Many agents which induce apoptosis are either oxidants or stimulators of cellular oxidative metabolism (Buttke and Sandstrom, 1994). For instance, tumor necrosis factor α (TNFα)-induced apoptosis was early shown to be dependent on ROS production. This was further supported by findings that antioxidants, such as *N*-acetylcysteine (NAC), and increased expression of SOD and thioredoxin were capable of protecting cells from TNF cytotoxicity. Subsequent studies have identified mitochondria as the main source of intracellular ROS production on treatment with TNF (reviewed in Shen and Pervaiz, 2006). Conversely, many inhibitors of apoptosis have antioxidant activity or enhance cellular antioxidant defenses. Being cysteine proteases, the caspases are sensitive to the redox state of the cell; hence, their activity can be blocked by excessive oxidative stress. Thus, alterations of intracellular redox state may either trigger or block the apoptotic death program, depending on the severity of the oxidative stress (Hampton and Orrenius, 1998).

In addition to Complex I and Complex III of the mitochondrial respiratory chain, a possible source of ROS generation in mitochondria might be the p66Shs protein, a redox enzyme that utilizes reducing equivalents of the mitochondrial electron transfer chain through the oxidation of cytochrome *c* (Giorgio et al., 2005). Redox-defective mutants of p66Shc are unable to induce mitochondrial ROS generation and swelling *in vitro* and are also resistant to mitochondrially-mediated apoptosis *in vivo*. Interestingly, a fraction of this cytosolic enzyme localizes within mitochondria, where it forms a complex with mitochondrial Hsp70. Upon stimulation of apoptosis, dissociation of this complex occurs, followed by the release of monomeric p66Shc and its interaction with cytochrome *c* to generate hydrogen peroxide. Conversely, reduction of p66Shc was shown to suppress oxidative damage in retinal pigmented epithelial cells and retina (Wu et al., 2006).

Redox regulation is an important factor affecting malignant cell survival. Modulation of the cellular redox balance via pharmacological stimulation of intracellular ROS production and/or depletion of protective reducing metabolites (such as glutathione and NADPH) may lead to oxidative stress and induction of apoptosis (Engel and Evens, 2006). A variety of therapeutic agents that modulate cellular redox systems including buthionine sulfoximine, ascorbic acid, arsenic trioxide, imexon, and motexafin gadolinium, can be used as single-agents or in combination for stimulation of apoptosis and, hence, killing of cancer cells.

The sensitivity of tumor cells to treatment with anticancer drugs depends on both the expression and function of antiapoptotic and antioxidant proteins. Antioxidant enzymes, such as catalase, Cu/Zn-superoxide dismutase (SOD1) and glutathione peroxidase (GPx1), protect cells against ROS. In addition to these enzymes, the peroxiredoxin (Prx) family members were shown to be frequently elevated in cancer, in particular in lung cancer cells. It has been shown that overexpression of Prx1 enhances the clonogenic survival of irradiated cells and suppresses ionizing radiation-induced c-Jun NH2-terminal kinase (JNK) activation and apoptosis (Kim et al., 2006).

Investigation of the role of the antioxidant protein PrxV demonstrated its ability to affect mitochondrial function. PrxV is a thioredoxin peroxidase, which is highly expressed in many tissues (Knoops et al., 1999). PrxV contains mitochondria- and peroxisome-targeting signals, directing its localization to mitochondria (Knoops et al., 1999) and peroxisomes (Kropotov et al., 1999; Yamashita et al., 2003). Clones of the lung carcinoma cell line U1810 with down-regulated expression of PrxV, or with its enzymatic function impaired (redox-negative PrxV), demonstrated increased sensitivity to treatment with the anticancer drugs etoposide and adriamycin. Pre-treatment of these clones with antioxidant *N*-acetyl-cysteine did not change their sensitivity to adriamycin, suggesting the involvement of a non-redox activity of PrxV. Conversely, overexpression of PrxV in multiple lung carcinoma cell lines increased cell radioresistance *in vitro*. Expression of the redox-negative PrxV mainly affected the mitochondrial pathway of apoptosis, as assessed

by cytochrome *c* release assay. Impairment of the PrxV enzymatic function also affected the mitochondrial membrane potential and calcium loading capacity of mitochondria, as well as mitochondrial morphology. Altogether, these findings suggest that PrxV is a multifunctional protein, which is essential for regulation of metabolic pathways induced by anticancer drugs (Kropotov et al., 2006).

Involvement of mitochondria in apoptosis

The biochemical machinery involved in apoptotic killing and dismantling of the cell can be activated by a variety of signals. Although distinct pathways leading to cell death are triggered by different signals, in many models of apoptosis they often merge at a common "regulator" of this multistep process, the mitochondria.

Mitochondria play a key role in the regulation of apoptotic cell death (Orrenius, 2004). Specifically, the release of some proteins from the intermembrane space of mitochondria is regarded as a key event in apoptosis induction. Among these proteins are cytochrome *c*, Apoptosis Inducing Factor (AIF), Smac/Diablo, Omi, etc. (Cai et al., 1998; Green and Reed, 1998). Once in the cytosol, cytochrome *c* interacts with its adaptor molecule, Apaf-1, resulting in the recruitment, processing and activation of pro-caspase-9 (Zou et al., 1999). Active caspase-9, in turn, cleaves and activates pro-caspase-3 and -7; these effector caspases are responsible for the cleavage of cellular proteins leading to biochemical and morphological features characteristic of apoptosis. AIF and the serine protease Omi, catalyze caspase-independent downstream events in the apoptotic process (Robertson et al., 2000a,b). Therefore permeabilization of the outer mitochondrial membrane (OMM) is considered a crucial event during the early phase of apoptosis.

Mechanisms of OMM permeabilization

Mitochondrial permeability transition

There are several potential mechanisms that may be responsible for the permeabilization of the OMM (Fig. 1). Hence, it may result from the induction of mitochondrial permeability transition (MPT) due to the opening of non-specific pores in the inner mitochondrial membrane (IMM), followed by osmotic swelling of the mitochondria, drop of the mitochondrial membrane potential, and rupture of the OMM, causing the release of intermembrane space proteins, including cytochrome *c* (Fig. 1A). MPT was described some thirty years ago by Haworth and Hunter in a series of seminal papers (Haworth and Hunter, 1979; Hunter and Haworth 1979a,b), in which they showed that Ca^{2+} uptake stimulates drastic changes in mitochondrial morphology and functional activity due to the opening of a non-specific pore in the mitochondrial inner membrane, commonly known as the MPT pore. The basic unit of the MPT pore is the VDAC-ANT-CyP-D (voltage-dependent anion channel-adenine

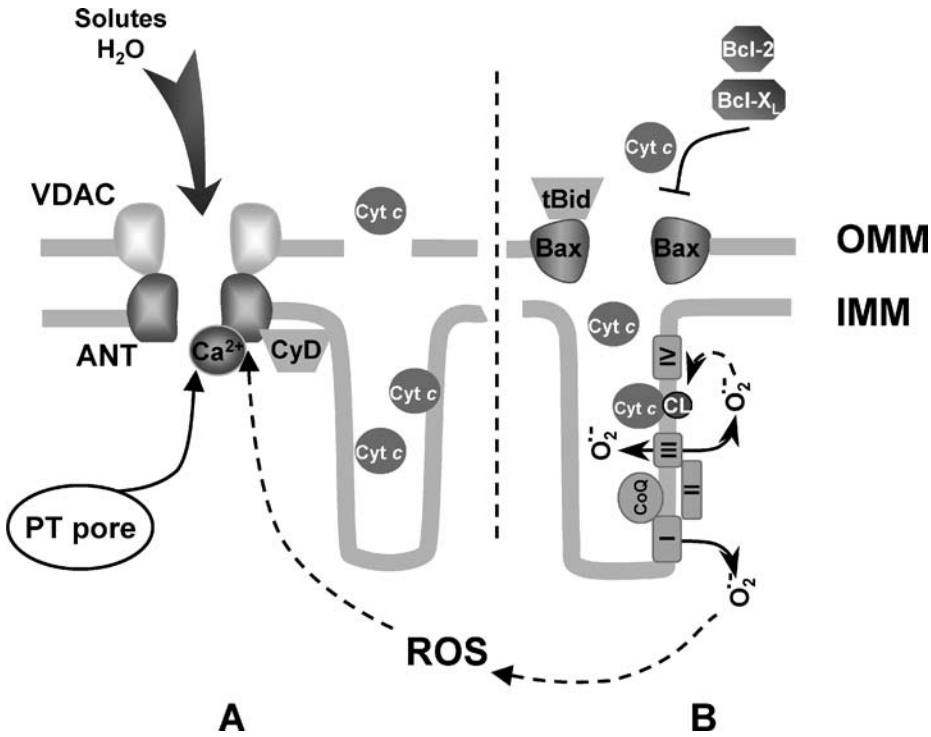

Fig. 1 Different pathways of cytochrome *c* release from mitochondria. **A** MPT-dependent release. Opening of the MPT pore results in mitochondrial swelling and rupture of the outer mitochondrial membrane. As a result cytochrome *c* is released. ROS, stimulated by anticancer drugs or generated in the mitochondrial respiratory chain, facilitate MPT induction via oxidation of SH groups in ANT. **B** Bax-mediated release of cytochrome *c*. Truncated Bid (tBid), promotes oligomerization of Bax (or Bak) and subsequent incorporation in the outer mitochondrial membrane. ROS facilitate cytochrome *c* detachment from cardiolipin. Antiapoptotic proteins, Bcl-2 and Bcl-X_L, prevent Bax-tBid interaction and release of cytochrome *c*

nucleotide translocase-cyclophilin D) complex located at contact sites between the mitochondrial inner and outer membranes. The VDAC-ANT complex is known to attract other proteins, in particular kinases (e.g., hexokinase or creatine kinase). Although Ca^{2+} is an obligatory component of MPT induction, this process can be facilitated by inorganic phosphate, oxidation of pyridine nucleotides, ATP depletion, low pH, and reactive oxygen and nitrogen species (ROS and NOS) (Crompton, 1999).

For many years, MPT was regarded as the prime mechanism responsible for the permeabilization of the OMM in apoptosis (Waring and Beaver, 1996; Yang and Cortopassi, 1998; Petit et al., 1998). However, ATP is required for the apoptotic process, and massive opening of pores in the inner membrane would lead to rapid ATP depletion. Since permeability transition is associated with the uncoupling of mitochondria, they would start hydrolyzing cytosolic ATP (uncoupling-stimulated ATPase activity). As a result, the intracellular ATP content would drop, causing a perturbation of cytosolic Ca^{2+} homeostasis and activation of various catabolic enzymes (proteases, phospholipases, etc). Hence, this model of mitochondrial outer membrane permeabilization may be most relevant during ischemia-reperfusion-induced cell death (Halestrap, 2006a,b). However, transient pore opening might also occur, whereby a small fraction of mitochondria would have open pores at a given time (Al-Nasser and Crompton, 1986); in this case, mitochondrial protein release would be expected to occur without observable large-amplitude swelling or drop in membrane potential of the entire organelle population. This process can be observed also under normal physiological conditions, especially in mitochondria located in close proximity to calcium "hot spots", microdomains, in which the local concentration of ionized calcium far exceeds the average concentration measured throughout the cytosol (Rizzuto et al., 1993). This local Ca^{2+} concentration might be high enough to induce Ca^{2+} overload and subsequent pore opening. Therefore, under the influence of toxic agents the frequency of such spontaneous pore opening and closure might increase, contributing to translocation of intermembrane space proteins into the cytosol.

In fact, opening of pores was shown to be a decisive event not only in necrosis, but also in many models of apoptosis, in particular, in apoptosis induced by TNF or Fas (Bradham et al., 1998; Shchepina et al., 2002), but not by staurosporine (Shchepina et al., 2002); in both apoptosis and necrosis in heart when induced by reperfusion (Argaud et al., 2005). Dopamine-induced apoptosis in the stunned heart (Nathan et al., 2005) was also shown to be mediated by MPT induction since Cyclosporin A (CsA), an inhibitor of pore opening, prevented apoptotic manifestations. FK-506, another potent calcineurin inhibitor, which, does not bind to cyclophilin, did not prevent cell death. Thus, stimulation of MPT is a powerful tool leading to cell death (apoptotic or necrotic) that can be used for cancer cell elimination.

Permeabilization of the OMM by Bcl-2 family proteins

The prime mechanism of OMM permeabilization during apoptosis involves members of the Bcl-2 family of proteins (Fig. 1B). The Bcl-2 family consists of more than 30 proteins, which can be divided into two groups: pro- and anti-apoptotic members. Importantly, a majority of these proteins fulfill their function at the level of mitochondria (Coultas and Strasser, 2003; Festjens et al., 2004). Overexpression of Bcl-2 was early found to prevent the efflux of cytochrome *c* from mitochondria as well as the initiation of apoptosis (Yang et al., 1997; Kluck et al., 1997). In contrast, Bax can stimulate cytochrome *c* release in different experimental systems. Bax-induced permeabilization of the OMM was shown to require the oligomeric form of Bax (Antonsson et al., 2000).

Oligomerization of Bax is a result of its binding to the truncated form of the proapoptotic protein Bid (tBid) (Eskes et al., 2000; Wei et al., 2000). Cells deficient in both Bax and Bak, but not cells lacking only one of these proteins, have been found to be resistant to tBid-induced cytochrome *c* release and apoptosis. Moreover, Bax- and Bak-deficient cells were also resistant to a variety of apoptotic stimuli that act through the mitochondrial pathway. Thus, activation of proapoptotic Bcl-2 family member, Bax or Bak, appears to be a predominant gateway to mitochondrial release of proteins required for cell death in response to diverse stimuli. Antiapoptotic proteins, e.g. Bcl-2, Bcl-X_L, Mcl-1, and Bcl-w, interact with the proapoptotic proteins, Bax and Bak, to prevent their oligomerization (Willis et al., 2005).

Recent observations revealed ROS involvement in Bax/Bak-dependent release of cytochrome *c*. It is known, that cytochrome *c* is normally bound to the outer surface of the IMM by both electrostatic and hydrophobic interactions with the unique mitochondrial phospholipid, cardiolipin, where it can reversibly interact with complexes III and IV of the respiratory chain. Hence, it seems that dissociation of cytochrome *c* from cardiolipin might be a critical first step in its release into the cytosol to trigger apoptosome formation and activation of the caspase cascade. In support of this assumption it was found that treatment of isolated mitochondria with oligomeric Bax was insufficient for substantial cytochrome *c* release, and that peroxidation of cardiolipin was an essential step in order to mobilize cytochrome *c* from the IMM (Fig. 1B). Based on these results, we proposed that cytochrome *c* release occurs by a two-step process that involves initial detachment of the hemoprotein from its binding to the IMM, followed by permeabilization of the OMM and its release into the extramitochondrial milieu (Ott et al., 2002). These findings suggest that cardiolipin plays an important role not only for the function and

interaction of components of the respiratory chain and related enzymes, but also for the retention of cytochrome *c* within the intermembrane space.

The two-step concept of cytochrome *c* release from mitochondria has been confirmed in several subsequent studies. For example, recent observations demonstrated that in the absence of complex I inhibitors, recombinant oligomeric Bax protein elicited only a minimal, not statistically significant, cytochrome *c* release (∼18%) from brain mitochondria. However, when the mitochondria were incubated with both recombinant Bax and complex I inhibitors, (which were shown to stimulate ROS production and, hence, cardiolipin oxidation) up to 65% of the mitochondrial cytochrome *c* was released. Thus, in accordance with the two-step concept, neither ROS production via complex I inhibition nor permeabilization of the outer membrane with Bax, alone, triggered extensive release of cytochrome *c*, whereas their combination resulted in a marked release (>60%) of this proapoptotic molecule (Piccotti et al., 2004). In another study, complex I dysfunction caused by either pharmacological or genetic means increased the releasable soluble pool of cytochrome *c* within the mitochondrial intermembrane space of isolated brain mitochondria. Upon mitochondrial permeabilization by Bax, more cytochrome *c* was released from brain mitochondria with impaired complex I activity (Perier et al., 2005).

Selective peroxidation of cardiolipin was shown to precede cytochrome *c* release in apoptotic cells (Kagan et al., 2005). It was demonstrated that a pool of cardiolipin-bound mitochondrial cytochrome *c* can catalyze cardiolipin peroxidation in the presence of hydrogen peroxide, which facilitates the detachment of cytochrome *c* from the IMM and its subsequent release into the cytoplasm. The peroxidase function of the cardiolipin-cytochrome *c* complex would be supported by the enhanced generation of ROS often observed during the early apoptotic phase and is compatible with the proposed two-step mechanism of cytochrome *c* release. It also provides a plausible explanation for the antiapoptotic effects reported for multiple mitochondrial antioxidant enzymes.

Interaction between the two pathways of cytochrome *c* release

Bcl-2 family proteins do not only regulate the permeabilization of the mitochondrial outer membrane via formation of pores, but can also modulate MPT induction. Thus, the presence of a higher level of Bcl-2 protein in mitochondria of Zajdela hepatoma was the cause of a delay in MPT induction as compared to liver mitochondria (Evtodienko et al., 1999). Conversely, it has been demonstrated that cell death resulting from Bax overexpression can occur via induction of MPT, since it was prevented by inhibition of the MPT with CsA in combination with the phospholipase A_2 inhibitor aristolochic acid (Pastorino et al., 1998). Furthermore, inclusion of recombinant oligomeric Bax in the incubation buffer markedly stimulated MPT induction in isolated rat liver mitochondria (Gogvadze et al., 2001).

Involvement of Bcl-2 family proteins in regulation of pore opening has been proposed to occur via interaction with VDAC (Shimizu et al., 1999). VDAC is known to be responsible for most of the metabolite flux across the mitochondrial outer membrane (Colombini, 1983). However, even in the open state it is not large enough (3 nm) to allow penetration of cytochrome *c* (14 kDa). On the other hand, Bax and Bak stimulated the opening of VDAC incorporated into liposomes and allowed encapsulated cytochrome *c* to pass, and the passage was prevented by Bcl-X_L (Shimizu et al., 1999). In contrast to this report, Rostovtseva and colleagues (2004) found no electrophysiologically detectable interaction between VDAC channels isolated from mammalian mitochondria and either monomeric or oligomeric forms of Bax. In contrast, another proapoptotic protein, tBid, proteolytically cleaved by caspase-8, affected the voltage gating of VDAC by inducing channel closure. The latter finding is in accordance with recent work by Colombini and colleagues, which also indicates that apoptosis is associated with VDAC closure, rather than opening, and that the resultant decrease in metabolite fluxes over the mitochondrial membranes leads to cytochrome *c* release and caspase activation (Rostovtseva et al., 2005). Hence, it appears that the role of VDAC in the permeabilization of the OMM during apoptosis requires further study.

Multiple mechanisms of cytochrome *c* release can coexist within one model of cell death. Thus, cytochrome *c* release triggered by low (up to 50 μM) doses of the anticancer drug arsenic trioxide was found to be Bax/Bak-dependent and, hence, was completely blocked in Bax/Bak double-knockout mouse embryonic fibroblasts. However, at higher arsenic concentrations cytochrome *c* release was caused by a direct effect of the toxicant on mitochondria resulting in MPT induction and occurred to a similar extent in both wild-type cells and cells lacking Bax and Bak (Nutt et al., 2005). Similar results were obtained in experiments with another anticancer drug – etoposide (Robertson et al., 2000a,b). Using Jurkat T-lymphocytes, a reconstituted cell-free system, or isolated liver mitochondria, etoposide was found to induce cytochrome *c* release via two distinct pathways. Caspase inhibition attenuated cytochrome *c* release triggered by a low dose (10 μM) of etoposide, but had no effect on cytochrome *c* release induced by higher doses of etoposide. Moreover, the higher dose of etoposide increased the sensitivity of Ca^{2+}-loaded isolated mitochondria to undergo permeability transition, an effect that was completely abolished by CsA. In contrast, MPT inhibitors did not block cytochrome *c* release seen with the lower concentration of etoposide (Robertson et al., 2000a,b).

Modulation of mitochondrial energetic state

In tumor cells the mechanisms normally involved in OMM permeabilization are suppressed. The level of antiapoptotic Bcl-2 family proteins is higher, thereby preventing membrane permeabilization by proapoptotic proteins (Bax, Bak). Mitochondria from cancer cells are more resistant to Ca^{2+} loading, therefore more Ca^{2+} is needed to stimulate MPT induction. Tumor cells as a rule overexpress antioxidant enzymes, in particular SOD, thus keeping ROS production at a low level. Sensitization of mitochondria in tumor cells might be an effective method to overcome apoptosis resistance. Can this be done with compounds that specifically affect mitochondrial function, for instance inhibitors of respiration?

In one of the first publications on this topic it has been shown that inhibitors of the mitochondrial respiratory chain, rotenone or antimycin A, and an inhibitor of mitochondrial ATP-synthase, oligomycin, induced apoptotic cell death in cultured human lymphoblastoid and other mammalian cells within 12–18 h. This was not caused by side effects of these compounds, since they did not induce apoptosis in cells depleted of mitochondrial DNA and thus lacking an intact respiratory chain. Apoptosis induced by respiratory chain inhibitors was not suppressed by antiapoptotic protein Bcl-2 (Wolvetang et al., 1994). Inhibitors of the mitochondrial respiratory chain were also shown to enhance cell sensitivity towards other apoptotic stimuli. Thus, Fas-mediated death of HeLa cells treated with respiratory inhibitors occurred at lower concentrations of Fas (Asoh et al., 1996).

On the other hand, neither inhibitors of the mitochondrial respiratory chain (rotenone or antimycin) nor an uncoupler of oxidative phosphorylation – carbonyl cyanide m-chlorophenylhydrazone (CCCP), were able to induce DNA fragmentation in freshly prepared thymocytes. In contrast, they clearly prevented DNA laddering induced by either glucocorticoids or the Ca^{2+} ionophore A23187 (Stefanelli et al., 1997; Galitovsky and Gogvadze, 1998), but not by staurosporine, or after treatment with mitogen (Stefanelli et al., 1997). Prevention of apoptosis was also observed in the presence of oligomycin. The antiapoptotic effect of oligomycin in cells treated with A23187 or etoposide was correlated to the inhibition of protein synthesis, while inhibition of apoptosis induced by dexamethasone, already evident at an oligomycin concentration of 10 ng/ml, was instead strictly correlated to the effect exerted on the cellular ATP level (Stefanelli et al., 1997). Suppression of apoptosis can be explained by the ability of these inhibitors to depolarize mitochondria and hence prevent Ca^{2+} accumulation and MPT, which in some cases is responsible for the release of cytochrome *c* and other apoptogenic proteins.

Surprisingly, the same inhibitors did not affect DNA fragmentation in cultured cells, i.e. human monocytic THP.1 or human leukemic U937 cells, but successfully abolished apoptotic changes in the plasma membrane (externalization of phosphatidylserine, PS), which are responsible for recognition, engulfment, and degradation of apoptotic cells by phagocytes. Traditionally, apoptotic changes in various cellular compartments are regarded as mechanistically linked events in a single "program", in which activation of caspases and proteolysis of intracellular substrates represent a final common pathway leading to cell death. Howerver, in human monocytic THP.1 cells triggered to undergo apoptosis, mitochondrial inhibitors dissociated plasma membrane changes from other features of apoptosis. These inhibitors blocked recognition of apoptotic cells by two classes of phagocytes but not activation of caspase-3, cleavage of poly(ADP-ribose) polymerase and DNA fragmentation. Thus changes governing clearance of apoptotic cells may be regulated by an independent pathway to those bringing about caspase activation (Zhuang et al., 1998). This finding could have important consequences for attempts to manipulate cell death for therapeutic gain *in vivo*.

PS exposure on the cell surface was shown to correlate with a decline in intracellular ATP level and inhibition of aminophospholipid translocase activity upon Fas stimulation (Gleiss et al., 2002). PS exposure requires the concomitant activation of a phospholipid scramblase and inhibition of an ATP-dependent aminophospholipid translocase. Thus, suppression of intracellular ATP levels by the glucose antimetabolite, 2-deoxyglucose, alone or in combination with glucose-free medium, potentiated Fas-induced PS exposure in the phospholipid scramblase-expressing Jurkat cell line and enables phospholipid scramblase-defective Raji cells to externalize PS in response to Fas ligation. These studies suggest that intracellular ATP levels can influence the externalization of PS during apoptosis, and implicate a role for the ATP-dependent aminophospholipid translocase in this process (Gleiss et al., 2002).

Undoubtedly, the disturbance of mitochondrial energetics via suppression of the mitochondrial respiratory chain, or mitochondrial uncoupling, will make mitochondria more vulnerable. One of the consequences of mitochondrial destabilization might be induction of MPT contributing to both apoptosis and necrosis. It is known that the mitochondrial membrane potential is among factors facilitating MPT, and a mild uncoupling of mitochondria was shown to stimulate pore opening (Scorrano et al., 1997). The effect of the respiratory chain inhibitors on Bcl-2-dependent cytochrome *c* release is less easy to explain. However, one could envision that inhibition of the respiratory chain should stimulate mitochondrial ROS production (Cadenas and Boveris, 1980), facilitating cytochrome *c* dissociation from cardiolipin. Indeed, a recent report clearly points to the involvement of ROS in rotenone-induced apoptosis (Li et al., 2003).

The consequences of the modulation of mitochondrial functional activity depend on many factors, such as cell type, nature of apoptotic stimulus and the role of mitochondria in cellular ATP supply. Nevertheless, mitochondrial inhibitors in combination with anticancer drugs seem to be a promising tool for cancer cell sensitization and elimination.

Mitochondria as a target for cancer cell killing

Stimulation of cell death plays a central role in regulation of tumor growth and tumor response to various forms of cancer therapy, including radiotherapy and chemotherapy. Numerous anticancer agents are able to trigger apoptosis in transformed or malignant cells by affecting mitochondrial functionality (Wang et al., 2005, 2006).

The importance of mitochondria as a target for anticancer treatment has been revealed in experiments with All-*Trans* Retinoic Acid (ATRA) (Schmidt-Mende et al., 2006). ATRA is a natural derivative of vitamin A, which is successfully used in the treatment of acute promyelocytic leukemia, where it induces terminal differentiation, and apoptosis of leukemic cells. Using the myeloid cell line P39, it was shown that ATRA disturbs mitochondrial function long before any detectable signs of apoptosis occur. These early changes included diminished mitochondrial oxygen consumption, decreased calcium uptake by mitochondria and, as a result, a lower mitochondrial matrix calcium concentration. Granulocyte Colony-Stimulating Factor (G-CSF) improved mitochondrial function – increased mitochondrial respiration and calcium accumulation capacity and blocked ATRA-induced apoptosis. Nifedipine, a plasma membrane calcium channel blocker, inhibited apoptosis-related changes, such as the loss of the mitochondrial membrane potential and activation of caspases (Schmidt-Mende et al., 2006). These results clearly show that the disturbance of Ca^{2+} homeostasis can sensitize mitochondria and facilitate cytochrome *c* release.

Similar mitochondrial targeting was also seen in the experiments with a human milk fraction containing **m**ultimeric **a**lpha-**l**actalbumin (MAL) (Kohler et al., 2001). MAL, a complex of a folding variant of alpha-lactalbumin and lipids was shown to kill cancer cells via apoptosis. Upon treatment of transformed cells, MAL localizes to the mitochondria and cytochrome *c* is released into the cytosol. This is followed by activation of the caspase cascade. Detailed investigation showed that addition of MAL to isolated rat liver mitochondria induced a loss of the mitochondrial membrane potential and mitochondrial swelling with subsequent permeabilization of the OMM and cytochrome *c* release. These changes were Ca^{2+}-dependent and were prevented by CsA. MAL also increased the rate of state 4 respiration in isolated mitochondria by exerting an uncoupling effect. This effect was due to the presence of fatty acid in the MAL complex, because it was abolished by BSA. BSA delayed, but failed to prevent, mitochondrial swelling as well as dissipation of the membrane potential, indicating that the fatty acid content of MAL facilitated, rather than caused, these effects. Similar results were obtained with HAMLET (**h**uman **a**lpha-lactalbumin **m**ade **le**thal to **t**umor cells), which is native alpha-lactalbumin converted in vitro to the apoptosis-inducing folding variant of the protein in complex with oleic acid (Kohler et al., 2001). In this model of apoptosis the presence of oleic acid in MAL can be considered as an additional factor causing a moderate uncoupling of mitochondria, sensitizing them and facilitating MPT.

Concluding remarks

Resistance of cancer cells to treatment is usually associated with deficiencies in various parts of the apoptotic machinery. Successful elimination of cancer cells, therefore, largely depends on the ability of anticancer drugs and treatments to stimulate silent apoptotic pathways. Mitochondria are promising candidates for such an approach. Release of proapoptotic proteins from mitochondria make them key participants in cell death execution. Effects on mitochondrial function by agents that suppress mitochondrial respiration and stimulate oxidative stress, or uncouple oxidative phosphorylation, can sensitize mitochondria in cancer cells and facilitate outer membrane permeabilization – "a point of no return" in many models of cell death.

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