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News and Commentary

Mitochondrial functions during cell death, a complex (I–V) dilemma

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Mitochondria, MOMP, and Caspase-independent Cell Death

During apoptosis in vertebrate cells, the process of mitochondrial outer membrane permeabilization (MOMP) appears to represent a point-of-no-return for many cell types (for a review, see Von Ahsen *et al*.[1] and Waterhouse *et al*.[2]). Several mechanisms for cytochrome c release have been proposed including disruption of the outer membrane, opening of the permeability transition pore and formation of pores for cytochrome *c* release (for a review see Von Ahsen *et al*.[1]). Recently Kuwana *et al*.[3] presented results strongly suggesting that MOMP requires neither the mitochondrial matrix, the inner membrane, nor mitochondrial proteins other than proapoptotic members of the Bcl-2 family. In this study, using a stepwise reductionist approach, we demonstrated that Bax, activated by Bid or by peptides corresponding to BH3 domains, was able to produce membrane openings sufficient to release high molecular weight dextrans. This permeabilization event was observed in mitochondria (to release cytochrome *c*), vesicles composed of purified mitochondrial outer membranes, vesicles composed of mitochondrial lipids, and vesicles composed of synthetic lipids, and in each case similar concentrations of Bax were effective. This required the mitochondrial lipid cardiolipin, and was inhibited by anti-apoptotic Bcl-x_L. These results suggest that the proapoptotic 'multidomain' members of the Bcl-2 family, such as Bax, are sufficient to produce MOMP. However, Scorrano *et al*.[4] suggested that most of the cytochrome *c* is sequestered in mitochondrial cristae and that the 'BH3-only' molecule tBID induces a striking remodeling of mitochondrial structure with mobilization of the cytochrome *c* stores (approximately 85%) in cristae. This remodeling appeared to require a mitochondrial permeability transition. Nevertheless, time-lapsed video microscopy and rapid filtration techniques have failed to provide evidence of a two-compartment model for cytochrome *c* release in intact cells.[5,6] In particular, in these studies, the rate of release of cytochrome *c*-GFP from mitochondria during apoptosis was observed to be temperature independent, suggesting that temperature-sensitive permeability transition and remodeling events do not play a prominent role in cytochrome *c* release, at least in the cells that were investigated.

With the exception of neurons,[7] most cells that release the proteins of the mitochondrial intermembrane space during apoptosis appear to be committed to die whether or not caspases are activated. In the presence of caspase inhibitors[8] or in cells lacking Apaf-1,[9] MOMP seems to be lethal, and although the cells die via a process that does not closely resemble apoptosis, antiapoptotic proteins such as Bcl-2, which block MOMP, also block the cell death.[10]

Similarly, death receptor signaling, although closely tied to caspase activation[11] and often dependent on caspase activation for death,[11] can apparently proceed to caspase-independent death.[12] One way in which this has been proposed to occur is via death receptor-induced changes in mitochondrial reactive oxygen species (ROS) generation.[13] Whether or not this also involves MOMP is currently unknown.

Why, though, does MOMP commit a cell to die? Here, we will consider three general answers, which are not mutually exclusive: (a) MOMP releases proteins that kill the cell, (b) MOMP destroys mitochondrial functions, and/or (c) MOMP induces ROS as a consequence of a and b.

Nasty Stuff from the Mitochondria

The prevailing explanation for caspase-independent cell death is that MOMP causes the release, from the mitochondrial intermembrane space, of proteins that kill the cell independently of caspases. Apoptosis-inducing factor (AIF),[14,15] Omi/Htra2,[16] and endonuclease G (Endo G),[17,18] have all been shown to have this capability in overexpression systems. In the case of AIF, injection of neutralizing polyclonal antibodies[19] and studies in AIF-null embryonic stem cells[20] have provided evidence that the release of AIF by MOMP is largely responsible for cell death. The roles of the other proteins have not been examined. In each case, the toxic protein performs at least one function that is independent of its possible role in cell death. This may be analogous to the dual functions of cytochrome *c*, or may indicate that these are not as important for caspase-independent cell death as we may believe.

However, some studies raise questions about the prevailing explanation. For example, HeLa cells that are maintained in caspase inhibitors and treated with apoptosis-inducing agents undergo MOMP and can persist for more than a week, provided they are given adequate glucose to maintain glycolysis. If, however, they are given pyruvate as a source of energy, MOMP proceeds as usual, but they maintain their viability only as long as they continue to generate ATP, dying within approximately 12 h of MOMP.[21] The death seen in

either case, however, is morphologically similar (Waterhouse and Green, unpublished observations). Thus, either glycolysis protects cells from the toxic effects of the death-promoting proteins released from the intermembrane space, or else other factors contribute to the death. We will consider these other factors below.

The idea that caspase-independent death may be inhibitable even after release of proteins from the intermembrane space comes from three sets of observations. First, neurons that are induced to undergo apoptosis by withdrawal of survival factors can recover upon readdition of these factors, even after cytochrome *c* release, provided that caspase activation is blocked.[7] This conclusion is based on the methods of detection of MOMP that rely on cell permeabilization, and therefore it is possible that the recoverable cells have not actually released the toxic intermembrane proteins. Further, only those cells maintaining $\Delta\Psi$m can recover.[7]

The second set of observations suggests that loss of apoptotic signaling downstream of MOMP can favor cellular transformation. Cells lacking Apaf-1 or caspase-9 are reportedly more easily transformed by oncogenes than wild-type cells[22] although there are concerns that these cell lines carried additional mutations (e.g., loss of p53). Nevertheless, many tumor lines lose Apaf-1 activity,[23] supporting the idea that this protein may act downstream of MOMP to suppress oncogenesis. If so, then at least some cells must recover and proliferate following MOMP.

Finally, at least one toxic protein released by MOMP can be regulated following this event. HSP70, which can act to block caspase-9 activation by the apoptosome,[24] can also block the activity of AIF.[25] Other mitochondrial intermembrane proteins that kill cells are enzymes that can therefore probably be regulated as well (e.g., Omi/Htra2 is a serine protease, EndoG is an endonuclease), but this has not been demonstrated.

What then are the other possible factors that contribute to caspase-independent death following MOMP? We consider these next.

Mitochondrial Power Outage

The mitochondrial transmembrane potential ($\Delta\Psi$m) is not only important for ATP production, but it is also required for mitochondrial protein import and to regulate metabolite transport. Protein import is particularly crucial for mitochondria. In fact, out of the hundreds of proteins present in these organelles, only 13 are encoded by the mitochondrial genome (which contain 37 genes: 13 encoding polypeptides, two for ribosomal subunits, and 22 for transfer RNA). All other mitochondrial proteins are encoded by nuclear genes and therefore must be imported from the cytosol. $\Delta\Psi$m is often used as an indication of cellular viability and this disruption has been implicated in a variety of apoptotic phenomena.[26–28] In addition, there have been numerous reports of impairment of the function of electron transport complexes in association with disorders like Parkinson's disease and Alzheimer's disease, conditions that are also associated with increases in markers of oxidative stress.[29]

The disruption of the $\Delta\Psi$m suggests that the proton-motive force and/or the inner membrane permeability has been affected during cell damage. In addition, a decreased rate of electron transfer will result in decreased consumption of mitochondrial pyruvate. In the absence of oxidative phosphorylation, pyruvate is converted to lactate and results in cytoplasmic acidification leading to cell death.[30]

Dissipation of the $\Delta\Psi$m is a general feature of apoptosis, irrespective of cell type (neurons, fibroblasts, thymocytes, monocytes, tumor cells, etc.) and of the apoptotic stimuli (drugs, ligation of death receptors, toxins, serum deprivation, etc.) (for a review, see Kroemer *et al*.[31]). The disruption of $\Delta\Psi$m during apoptosis is also observed in cells lacking mitochondrial DNA (ρ°cells), which as a consequence have impaired OXPHOS.[28] While there is controversy regarding the meaning of the loss of $\Delta\Psi$m during apoptosis (e.g., whether this is a cause or an effect of outer membrane permeabilization), the consequences are predictable. The drop of $\Delta\Psi$m during apoptosis is expected to induce a cessation of the import of most proteins synthesized in the cytosol, release of Ca^{2+} and glutathione from the mitochondrial matrix, uncoupling of oxidative phosphorylation with cessation of ATP synthesis, oxidation of $NAD(P)H_2$ and glutathione, and finally hyperproduction of superoxide anion by the uncoupled respiratory chain. These events can all participate in the demise of the cell. Nevertheless, dissipation of $\Delta\Psi$m by addition of protonophores to cells in culture does not necessarily cause cell death; we have observed that treated cells can survive for long periods of time (and proliferate) provided that ROS are scavenged (Waterhouse and Green, unpublished observations). It may be, therefore, that loss of $\Delta\Psi$m, *per se*, is not necessarily lethal, at least in the short term. However, the events responsible for it may be.

Using single-cell analysis Waterhouse *et al*.[21] demonstrated that a reduction in $\Delta\Psi$m followed within minutes after the release of cytochrome *c*, but in the absence of caspase activity, mitochondria use cytochrome *c* at the concentration maintained within the cytoplasm to regenerate $\Delta\Psi$m and maintain ATP generation (for hours or days depending on the cell type and the mode of induction). This maintenance of $\Delta\Psi$m suggests that if the cell can repair mitochondria, it may be rescued from subsequent death. This will depend on precisely why cells that have released cytochrome *c* eventually die, even if downstream caspases are blocked. Cell death under these conditions appears to follow a drop in ATP levels (see above), suggesting that the processes are linked. However, this is only correlative at present.

Loss of mitochondrial energy production will lead to cell death unless another source of energy is available to the cell. Apoptosis is an active (ATP-requiring) process and if sufficient ATP is not present, the cell death deviates from an apoptotic to a necrotic phenotype.[32] This finding could explain the frequent appearance of necrotic cells mixed with apoptotic cells in the center of solid tumor or in ischemic nervous tissues,[33] areas where the blood flow is very limited, so that the rapid consumption of glucose leads to a dramatic decrease in ATP, resulting in an inhibition of apoptosis and an induction of necrotic cell death.[32]

It has been suggested that the disruption of mitochondrial ATP/ADP exchange may contribute to the initiation of apoptosis.[34] The exchange of matrix ATP for cytosolic ADP is dependent on the two protein complexes mainly composed

of adenine nucleotide transporter (ANT) in the inner membrane and voltage-dependent anion channel (VDAC) in the outer membrane (Figure 1). Inhibition of ANT function or closure of VDAC could result in a lack of available ADP in the mitochondrial matrix, leading to the inhibition of ATP production and to cell death. Under normal physiological conditions, VDAC exists in an open configuration that permits the exchange of anionic metabolites across the outer membrane.[35] However, in this physiological open state, VDAC is not permeable to cytochrome *c*. Bcl-2 members can interact with membranes making them permeable to ions, small molecules, and possibly to proteins as well. A modification in VDAC opening is predicted to have profound effects on cellular bioenergetics and cell death and a direct action of Bcl-2 members on VDAC conformation has been described. For example, Shimizu *et al.*[36] described the ability of Bcl-xL to regulate the gating of VDAC *in vitro*. In addition, Vander Heiden *et al.*[34] provided evidence that Bcl-xL can prevent cell death by facilitating mitochondrial ATP/ADP exchange through VDAC. Although the observations by these groups are conflicting, these results suggest that Bcl-2 and Bcl-xL are able to interact with outer membrane channels (including VDAC) leading to the stabilization of their physiological configuration (whether open or closed is not agreed upon). Shimizu *et al.* suggested that proapoptotic Bcl-2 members (Bax and Bak) disrupt the integrity of VDAC, allowing it to adopt a nonphysiological open state that permits the release of proteins present in the intermembrane space, including cytochrome *c*. According to these authors, the ability of Bcl-2 proteins to regulate the open state and integrity of VDAC could account for their ability to be either anti- or proapoptotic. Nevertheless, the action of proapoptotic Bcl-2 members on VDAC conformation is still controversial. In fact, many groups have failed to validate the requirement of either VDAC[37,38] or ANT[39,40] for Bax killing, as originally described.[3]

In conclusion, regardless of the exact mechanism leading to permeabilization of the outer membrane (nonspecific channel, permeability transition, and/or outer membrane rupture), once the electron transport chain is impaired (as a consequence of cytochrome *c* release and/or lack of available ADP), the loss of mitochondrial energy could lead to cell death (dependent or not on caspases). Alternatively, the loss of $\Delta\Psi$m also disrupts

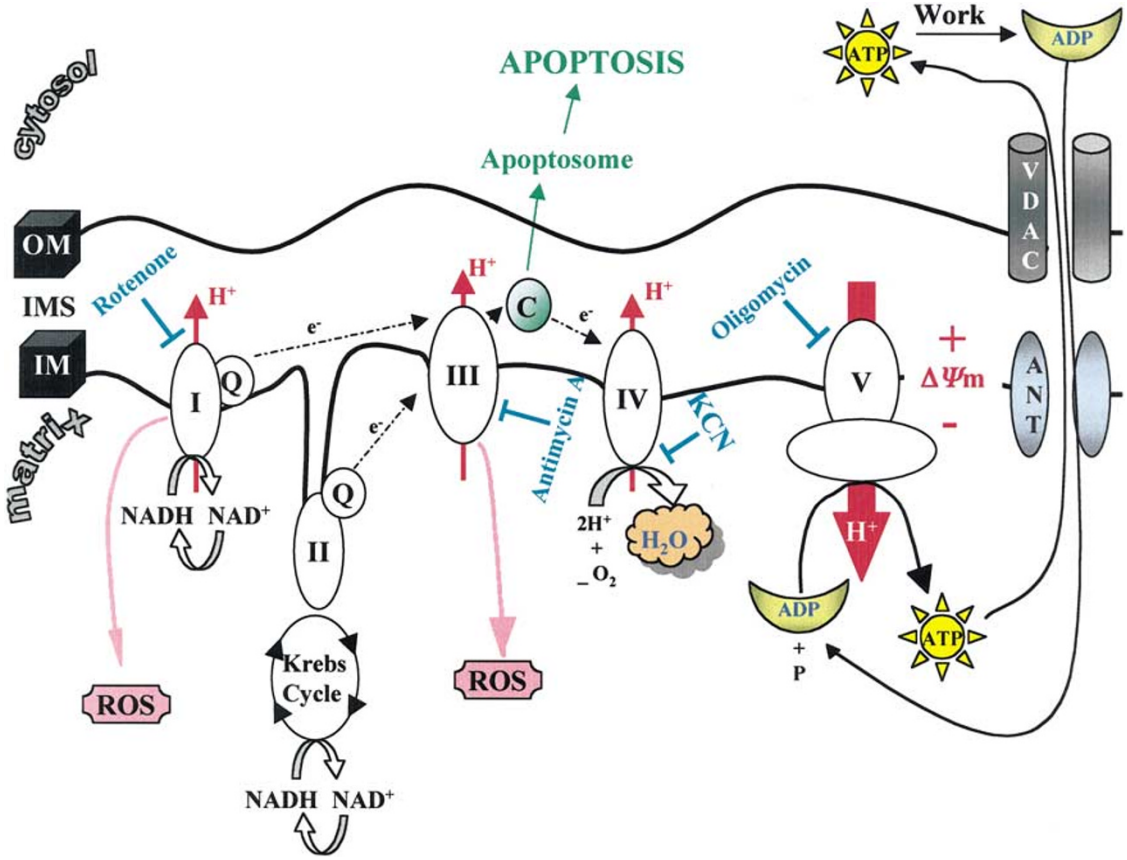

Figure 1 Electron transport chain, ATP generation, and ROS production. Different complexes (I–V) of the electron transport chain are represented in the inner membrane (IM) of the mitochondria. Complexes I and II are where electrons enter this chain. Complex II uses the conversion of succinate to fumarate (produced by the Krebs cycle) to transfer an electron to coenzyme Q (Q). Cytochrome *c* (C) transfers those electrons from complex III to complex IV. On acceptance of the electron, complex IV then converts H^+ and O_2 to water. Except for complex II, complexes from I to IV pump protons out of the matrix to the intermembrane space (IMS). This charge distribution, the mitochondrial transmembrane potential ($\Delta\Psi$m), is used by complex V (ATP-synthase) to convert ADP into ATP. ATP is then released into the cytosol via the adenine nucleotide transporter (ANT) and the voltage-dependent anion channel (VDAC). Once cytosolic, ATP is converted to ADP during ATP-dependent processes in the cell and re-enters the mitochondrial matrix. ROS (reactive oxygen species) that are produced by this process are indicated.Several respiratory inhibitors are represented: rotenone: complex I inhibitor; antimycin A: complex III inhibitor; potassium cyanide (KCN): complex IV inhibitor, and oligomycin: complex V inhibitor.

other mitochondrial functions, including protein transport into the matrix, and it may be that this (rather than loss of energy production) is the lethal hit. Finally, the disruption of the outer membrane may be the lethal event, irrespective of changes in $\Delta\Psi$m, for reasons that remain unclear.

ROS: Burning Down the House

In addition to their critical role in ATP synthesis, mitochondria are also the major source of ROS and mitochondria are the first compartment in the cell that is damaged by these ROS. It is widely believed that ROS contribute to the pathogenesis of a number of neurodegenerative diseases and other pathological states.[29] Exposing cells to oxidative stress has been shown to induce lipid peroxidation, calcium mobilization, mitochondrial permeability transition, ATP depletion, protein oxidation, loss of electron transport, and DNA damage, all of which may impact on the death of a cell. In addition, antioxidants and thiol reductants such as *N*-acetyl cysteine (NAC),[41] overexpression of thioredoxin,[42] and manganese superoxide dismutase (MnSOD)[43]can apparently block or delay apoptosis in some cases.

ROS are generated by incomplete reduction of molecular oxygen during the process of oxidative phosphorylation. It has been suggested that during the transfer of electrons, 1–5% will 'escape' the OXPHOS system and participate in the formation of superoxide. In turn, superoxide is converted by MnSOD to H_2O_2, which is more stable and more readily released by mitochondria. In stressful conditions, the ROS production could overwhelm the detoxication pathways and contribute to cell death.

The mechanisms responsible for the generation of ROS by the electron transport chain have been extensively investigated, primarily in mitochondria derived from heart muscle[44] (see Degli Esposti[45] for a review of methods to measure mitochondrial ROS). The principal source appears to be the redox cycling ubiquinone in complex III[46] (see Figure 1). An additional source of superoxide is complex I as a result of reverse electron transfer at high membrane potential values.[44] Of note, the role of complex II in mitochondrial ROS has often been neglected owing to the effects of antimycin, which invariably enhances ROS production.[44,47,48] In fact, the contribution of complex III to the basal production of mitochondrial ROS is relatively small in comparison with that of complex II,[49] especially in cultivated cells. Interestingly, the level of ROS produced via complex I or complex II depends on the cell type.[50,51] For example, rat brain mitochondria respiring on NADH-linked substrates (complex I) produce a very small ROS signal in the absence of electron transport chain inhibitors, but mitochondria derived from other species and tissues (like mouse heart, kidney, brain[52]) show a very high production under the same conditions. The basis for these differences remains unclear.

A role of ROS during apoptosis was initially proposed based upon the observation that Bcl-2, a general inhibitor of apoptosis in mammalian cells, has an apparent antioxidant function.[53] It was unclear whether the protective effect was due to scavenging function or to a regulation of the production of ROS.[53,54] Cai and Jones[55] demonstrated that mitochondria from cells overexpressing Bcl-2 had no increase in superoxide production following staurosporine treatment. In addition, if the same cells were treated with cyanide (complex IV inhibitor), this resulted in ROS generation at a rate similar to that of control cells. Thus, the effect of Bcl-2 was not due to a direct electron-scavenging or superoxide-metabolizing activity of Bcl-2 itself but rather to the prevention of ROS production. We now believe that the ability of Bcl-2 to block ROS production is via the inhibition of outer membrane permeabilization.

In addititon, it has been established that a substantial inhibition of respiration is required to observe increased ROS production, indicating the existence of a broad margin of safety from the mitochondrial machinery. In theory, an inhibition of ATP synthesis could generate a sufficiently hyperpolarized state to support succinate-driven ROS generation. We have previously shown that ATP production after cytochrome *c* release can be maintained provided caspase activation is blocked.[21] More recently, we have shown that the rapid loss of $\Delta\Psi$m and the generation of ROS occurring during cell death are because of the effects of activated caspases on mitochondrial electron transport complexes I and II. Caspase 3 is able to disrupt oxygen consumption induced by complex I and II substrates (but not by electron transfer to complex IV). Similarly, $\Delta\Psi$m generated in the presence of complex I or II substrates is disrupted by caspase-3 and ROS are produced. In addition, we have shown that caspase feedback on mitochondria is dependent upon permeabilization of the organelle (because of MOMP), and then the caspases act on both complexs I and II to disrupt oxygen consumption and $\Delta\Psi$m, and to induce ROS production.[56]

Interestingly, under some conditions, ROS can stimulate protective mechanisms that block cell death.[57,58] For example, ROS production can lead to activation of NF-κB and cell survival. Furthermore, caspases can be inhibited by oxidants.[59] In the absence or near absence of O_2, proapoptotic stimuli can function to induce apoptosis,[60] and in some cases sensitivity is enhanced. Therefore, not only can ROS promote cell death, they can also inhibit it. Together, these observations suggest that ROS is a regulator but not a mediator of apoptosis. The extent to which ROS contribute to caspase-independent cell death, however, remains an open question.

Conclusions

It is well established that after cytochrome *c* release and in the absence of caspase activity, mitochondria retain enough function to allow cell survival. Nevertheless, the absence of caspase activity does not inhibit cell death (leading to a caspase-independent death). As a consequence, when mitochondria are damaged apparently two parallel ways of killing the cells take place: one is quick and dependent on caspase activation and the other is slower and may be caused by a major dysregulation of mitochondrial functions. In each form of death, mitochondria play central roles, and may be the decision point at which cell life or death is determined. The regulation of this critical step, both during the process of apoptosis and by our attempts to manipulate it pharmacologically, can provide powerful ways to control cell survival.

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