We are, indeed, in urgent need to understand more precisely cell death. The therapeutic suppression of cell death is still in its infancy, even in conceptual terms, irrespective of the advancing knowledge on the morphological appearance of pathological cell death occurring in our body. Cytoprotection by suppression of cell death should be the therapeutic goal of organ preservation as well as of the clinical management of major diseases, including stroke, infarction and neurodegeneration. However, so far very few strategies for cytoprotection have proven successful, even in animal models of acute cell loss. Similarly, we still anxiously await the development of chemotherapeutics that would elicit immunogenic cell death, in spite of the fact that there has been some success in developing cell death-inducing regimens for cancer chemotherapy.[41,42] Such an 'immunogenic chemotherapy' would allow for the immune system-mediated eradication of tumor (stem) cells that resist cell death induction, thereby increasing the efficacy of treatment and the probability of total remission. These examples illustrate how an exhaustive investigation of the mechanisms underlying cell death could have enormous impacts on human medicine.

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Role of cardiolipin in cytochrome *c* release from mitochondria

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Mitochondria play a pivotal role in the regulation of apoptotic cell death as well as in several cellular metabolic processes, including energy supply.[1,2] The latter is achieved by oxidative phosphorylation of ADP to ATP using the electrochemical

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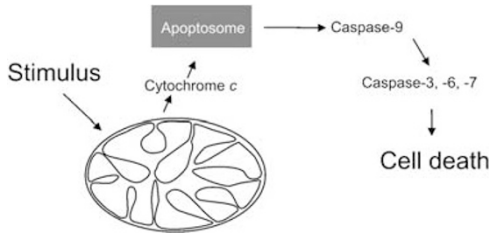

Figure 1 Mitochondrial control of caspase activation. Proapoptotic stimuli induce the release of cytochrome *c* from mitochondria. In the cytosol, cytochrome *c* participates in apoptosome formation, which results in activation of caspase-9, allowing subsequent activation of the executioner caspases-3, -6 and -7 responsible for the dismantling of the cell during apoptosis

proton gradient generated by the stepwise transport of electrons from oxidizable substrates to molecular oxygen mediated by the mitochondrial respiratory chain. One component of the respiratory chain is cytochrome *c*, which transfers electrons from Complex III to Complex IV. In apoptosis signaling, however, this vital function of cytochrome *c* is gradually lost.[3] Once the outer mitochondrial membrane (OMM) has been permeabilized by proapoptotic members of the Bcl-2 family of proteins, cytochrome *c* is released from the mitochondrial intermembrane space into the cytosol. Here, it triggers apoptosome formation and the activation of the caspase cascade, which leads to the cleavage of a host of cellular proteins and dismantling of the cell (Figure 1). Within the mitochondria, cytochrome *c* is bound to the outer surface of the mitochondrial inner membrane (IMM) by its association with cardiolipin, an anionic phospholipid present predominantly in the mitochondria. We and others have previously suggested that the interaction of cytochrome *c* with cardiolipin critically determines the amount of the hemoprotein that can be released during apoptosis signaling.[4,5] Furthermore, there is emerging evidence that proapoptotic Bcl-2 family proteins might require cardiolipin for permeabilization of the mitochondria during apoptosis.[6] However, the precise role of cardiolipin in the release of cytochrome *c* from mitochondria during apoptosis is still unclear and is the subject of this commentary.

Interaction of Cytochrome *c* with Cardiolipin

Cardiolipin is an anionic phospholipid that is present in the mitochondria, more specifically, in the IMM where it is also synthesized. Because of its unique structure among phospholipids, cardiolipin confers fluidity and stability to the IMM and is also required for the function of several IMM proteins, for example cytochrome oxidase and the adenine nucleotide translocator. In addition, cardiolipin was recently found to be required for the organization of the respiratory chain into supramolecular assemblies.[7] Cytochrome *c*, a component of the respiratory chain, is normally attached to the outer surface of the IMM in a loosely and tightly bound way,[8] mainly by an association with cardiolipin (Figure 2). The molecular interaction between cardiolipin and cytochrome *c* involves electrostatic interactions at the A-site of the hemoprotein, whereas hydrophobic interactions and hydrogen bonding take place at its C-site.[9] To explain the latter binding mode, it was postulated that one of the acyl chains of cardiolipin may be inserted into a hydrophobic pore in cytochrome *c,* whereas the others extend into the phospholipid bilayer.

The mitochondrial respiratory chain is also the main source of reactive oxygen species (ROS) in most aerobic cells, and cardiolipin is a prime target of oxidative damage not only because of its proximity to the site of ROS generation but also because of its highly unsaturated acyl chains. It was earlier found that cardiolipin oxidation decreases its binding affinity for cytochrome *c* and, more recently, that oxidative modification of cardiolipin facilitates cytochrome *c* mobilization from the IMM.[4,10–12] On the basis of these results we hypothesized that cytochrome *c* release during apoptosis occurs by a two-step process, involving first the detachment of the hemoprotein from the outer surface of the IMM, followed by permeabilization of the OMM and the release of cytochrome *c* into the extramitochondrial milieu (Figure 2).[4] These findings indicate that cardiolipin plays an important role not only in mitochondrial energy metabolism, but also in the retention of IMM-bound cytochrome *c* within the intermembrane space.

Accumulating data suggest that a decrease in cardiolipin content in the IMM correlates with a similar decrease in the amount of membrane-bound cytochrome *c* in the mitochondria. Hence, ROS-mediated cardiolipin peroxidation has been experimentally shown to cause detachment of bound cytochrome *c* from the IMM in both *in vitro* and *in vivo* models (for review see Orrenius *et al.*[13]). Furthermore, selective peroxidation of cardiolipin was recently demonstrated by Kagan *et al.*[5] to precede mitochondrial cytochrome *c* release during apoptosis. Searching for the mechanism of cardiolipin oxidation, the authors found that cytochrome *c*, in complex with cardiolipin, catalyzes H_2O_2-dependent cardiolipin peroxidation which, in turn, triggers the detachment of cytochrome *c* from its binding to the outer surface of the IMM and its subsequent release into the cytosol through pores in the OMM (Figure 2). Conversely, a host of recent studies have shown a correlation between preserved cardiolipin content and resistance to apoptosis upon manipulation of various mitochondrial antioxidant enzymes, including peroxiredoxin III, glutaredoxin 2 and glutathione peroxidase 4.[12,14,15] In addition, phospholipase A2-mediated degradation of cardiolipin,[16] or calcium-induced detachment of cytochrome *c* from cardiolipin binding,[17] have been proposed as other mechanisms that interfere with cytochrome *c* binding to the IMM (Figure 2).

The two-step concept of cytochrome *c* release from mitochondria during apoptosis has now been supported by several subsequent studies. For example, it was recently demonstrated that recombinant, oligomeric Bax protein triggered only minimal cytochrome *c* release ($\sim$18%) from brain mitochondria in the absence of Complex I inhibitors.[18] 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. These data suggest that the interaction between cytochrome *c* and cardiolipin critically limits the amount of cytochrome *c* that is released during apoptosis signaling, and that modulation of cardiolipin leading to a decreased binding affinity for cytochrome *c* is a critical early step in this process.

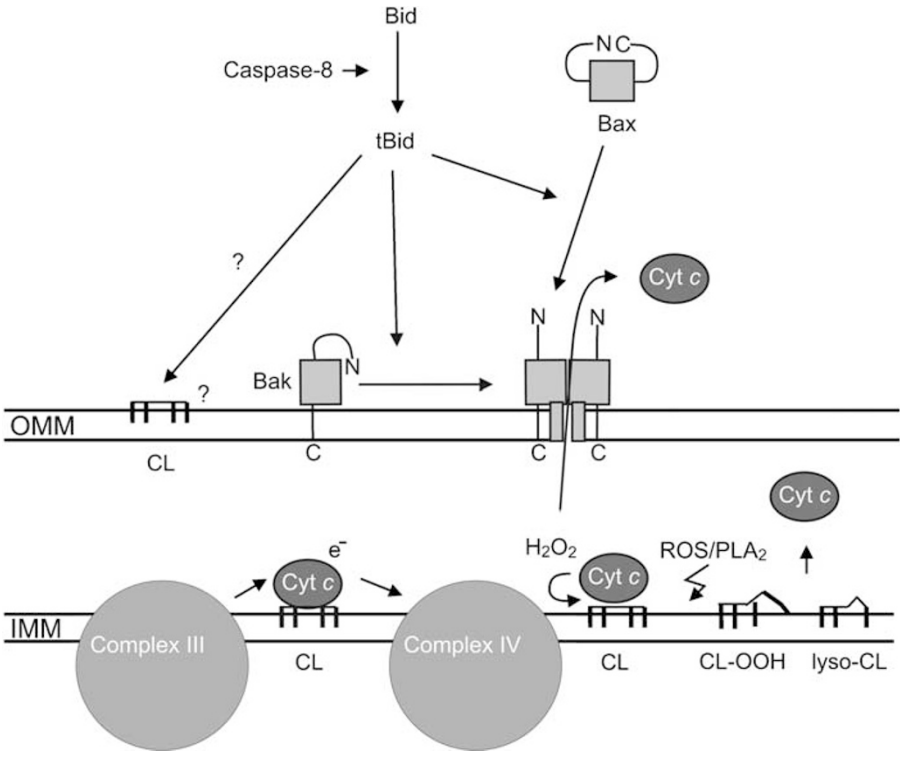

Figure 2 tBid-induced cytochrome *c* release from mitochondria. tBid, formed by proteolytic cleavage of Bid, for example by caspase-8, might exert multiple effects at the level of mitochondria. Hence, tBid can induce insertion and oligomerization of Bax in the OMM. This leads to the formation of pores that allow the release of cytochrome *c* (Cyt *c*) into the cytosol. tBid can also directly induce oligomerization of membrane-associated Bak leading to pore formation. In addition, tBid might interact with cardiolipin to influence its distribution between the mitochondrial membranes or induce changes in the ultrastructure of mitochondria. Cytochrome *c* is normally bound to the IMM by cardiolipin (CL) and transports electrons from Complex III to Complex IV. During apoptosis, cytochrome *c* detaches from cardiolipin and appears as a soluble protein in the intermembrane space. Detachment of cytochrome *c* from cardiolipin can be mediated either by direct formation of oxidized cardiolipin (CL-OOH) induced by the cytochrome *c*-cardiolipin peroxidase, or through oxidative modification of cardiolipin by ROS originating from the respiratory chain or by phospholipase-A_2 (PLA_2)-mediated formation of lyso-cardiolipin (lyso-CL)

Is There a Role for Cardiolipin in tBid/Bax-Induced Cytochrome *c* Release?

The mechanism(s) of permeabilization of the OMM during apoptosis signaling has been studied extensively in recent years. One major pathway involves the proapoptotic Bcl-2 family proteins. The Bcl-2 family includes three different groups of proteins: (a) antiapoptotic proteins, for example Bcl-2 or Bcl-X_L, which reside permanently in the mitochondria and, partly, in the endoplasmic reticulum; (b) proapoptotic proteins, notably Bax and Bak, which are responsible for the permeabilization of the OMM during apoptosis signaling; and (c) proapoptotic effector proteins of the BH3-only group, like BIM, Puma, Noxa and Bid. The ratio of proapoptotic to antiapoptotic Bcl-2 family proteins critically determines the susceptibility of a cell to undergo apoptosis.

Upon activation by distinct signals, BH3-only proteins target either the proapoptotic proteins of the Bax type to induce their oligomerization (Bid, Bim and Puma) or the antiapoptotic proteins of the Bcl-2 type to block their inhibitory action on the Bax-like factors (BAD, Bik and Noxa).[19] One pathway that has been studied in particular detail involves the cytoplasmic protein Bax and the BH3-only protein Bid. Cleavage of Bid by multiple proteases, including caspase-8, yields a truncated 16 kDa form, termed tBid.[20] This fragment can then promote the insertion of Bax into the OMM and its subsequent oligomerization (Figure 2).[21] The oligomeric form of Bax is believed to form a pore that allows the extrusion of several intermembrane space proteins into the cytosol. Oligomeric Bax displays an altered structure, including the exposure of a N-terminal domain[22] and insertion of the C-terminal domain and of the central, pore-forming α-helices 5 and 6 into the membrane.[23] The C terminus is of critical importance for the proapoptotic feature of Bax[22] and has some similarities to the signals that normally direct tail-anchored proteins to their target membrane. However, the activation of Bax and Bak might not be the only outcome of tBid generation. Hence, it was reported that tBid induces a perturbation of mitochondrial ultrastructure, resulting in the mobilization of the pool of cytochrome *c* residing in the intercristal space created by the invaginations (the cristae) of the IMM (Figure 2).[24] Whether the effect is a direct result of the action of tBid on mitochondria, or whether this remodeling might reflect changes in mitochondria induced by the release of cytochrome *c*, remains unclear. Moreover, another study has suggested that tBid might also cause changes in the distribution of phospholipids, notably cardiolipin, between the OMM and the IMM (Figure 2).[25] An increased cardiolipin content in the OMM during apoptosis has been reported in some systems.[26]

Two different models for the role of tBid in Bax activation have been proposed. Firstly, tBid itself might bind to the mitochondria and thereby provide a high-affinity binding site for Bax, which would help targeting this molecule to the mitochondria. In

support of this hypothesis, it was recently shown that a membrane-targeted BH3-domain of Bid could potently activate Bax to permeabilize artificial liposomes.[27] In addition, it was reported earlier that cardiolipin allows the specific targeting of tBid to mitochondria.[28] Secondly, tBid could help Bax undergo the conformational changes required for its insertion and oligomerization in the OMM. In contrast to Bax, Bak, which can also mediate mitochondrial cytochrome *c* release, is constitutively present in the OMM and does not require targeting to the mitochondria during apoptosis. In this case, the role of tBid could be to mediate the conformational changes resulting in the assembly of Bak oligomers in the OMM.[29]

A requirement of cardiolipin for mitochondrial cytochrome *c* release was first suggested by studies showing that cardiolipin was obligatory for Bax-mediated pore formation in liposomes.[6,30] Specifically, Kuwana *et al*.[6] used reconstituted membranes and/or synthetic liposomes encapsulating fluorescently labeled dextran molecules, to demonstrate that Bax-induced dextran release required the presence of cardiolipin in the liposomes. tBid, or its BH3-domain peptide, was found to be able to activate monomeric Bax to produce membrane openings that allowed the passage of very large (2 MDa) dextran molecules, mimicking the translocation of mitochondrial proteins during apoptosis. This process required cardiolipin and was inhibited by antiapoptotic Bcl-X_L. Thus, the authors concluded that mitochondrial protein release during apoptosis might be mediated by supramolecular openings in the OMM, promoted by BH3/Bax/lipid interaction and directly inhibited by Bcl-X_L.[6] However, it is still uncertain exactly how the protein–lipid interaction might lead to formation of the huge pores in the OMM. Moreover, it is difficult to understand why such pores would not also be formed in the IMM, in which the content of cardiolipin is much higher than in the OMM. Furthermore, the finding of cardiolipin in the OMM was based solely on mitochondrial subfractionation studies. Dependent on the purity of the preparation and origin of mitochondria, cardiolipin is normally found to be either not present, or present in very minor quantities, in the OMM fraction.[31]

To investigate further a possible role for cardiolipin in OMM permeabilization, we have compared cytochrome *c* release from cardiolipin-deficient and wild-type yeast mitochondria.[32] It was found that neither the mitochondrial association of exogenous, recombinant Bax, nor the resulting cytochrome *c* release, was dependent on the cardiolipin content of the yeast mitochondrial membranes. In these experiments, Bax associated equally well with both wild-type and cardiolipin-deficient mitochondria under conditions that led to the release of cytochrome *c* from both types of mitochondria. However, we did find that cytochrome *c* was bound more 'loosely' to the cardiolipin-deficient IMM compared to the wild-type control.[32] These observations are in accordance with a recent study demonstrating that cardiolipin is not required for the killing of yeast cells by the overexpression of Bax.[33]

Decreased Cardiolipin Content Facilitates Cytochrome *c* Detachment from the IMM and Promotes Apoptosis

Hence, it appears that cardiolipin might have two functions in apoptosis signaling, namely (a) to offer a high-affinity binding site on the mitochondria for tBid and (b) to decrease the pool of free cytochrome *c* in the intermembrane space by promoting the association of the hemoprotein with the IMM. In the first case, a decreased cardiolipin level should result in a lesser ability of tBid/Bax to release cytochrome *c* from the mitochondria and, hence, in the inhibition of cell death. In the second case, decreased cardiolipin content in the IMM would result in a larger pool of free cytochrome *c* ready to be released from the mitochondrial intermembrane space and accelerated cell death.

Recently, Choi *et al*.[34] addressed the question of the role of cardiolipin in apoptosis signaling using RNAi knockdown of the mammalian cardiolipin synthase to decrease the content of this phospholipid in the mitochondria. When exposing the cells to triggers of apoptosis, such as agonistic Fas antibody or TNFα, it was found that decreasing the cardiolipin level to 25% of that present in control cells led to accelerated cell death. Apparently, cell death induced by the surface receptor-mediated pathway, which involves caspase-8-mediated Bid cleavage and Bax oligomerization in most cell types, was accelerated, rather than inhibited, by reduction of the cardiolipin level. Further investigation of the molecular basis for the accelerated death rate in cells with decreased cardiolipin content revealed that free cytochrome *c* was present in higher quantities in the mitochondrial intermembrane space and could therefore be released more efficiently during cell death signaling. A similar correlation between lowered cardiolipin content and release of cytochrome *c* from the IMM has previously been found in palmitate-induced apoptosis of cardiomyocytes.[35] Growing cells in the presence of this fatty acid in the culture medium resulted in a decreased mitochondrial cardiolipin content and a compensatory increase in its biosynthetic precursor, phospatidylglycerol, as well as enhanced rate of cell death. In contrast, cultivating cells in the presence of an unsaturated fatty acid, oleate, did not cause a decrease in cardiolipin content, provoke cytochrome *c* release or affect death rate in cardiomyocytes.

Concluding Remarks

The repeated observation that a reduced content of cardiolipin decreases cytochrome *c* binding to the IMM and facilitates mitochondrial cytochrome *c* release and apoptotic cell death is in accordance with the proposed two-step hypothesis.[4] In contrast, these findings do not support a prominent role for cardiolipin in targeting tBid to the OMM, as the association of tBid with mitochondria was unchanged by the reduced cardiolipin level.[34] Similarly, although a requirement of cardiolipin for tBid/Bax-induced cytochrome *c* release was demonstrated with artificial liposomes, this has not yet been found to be the case in experiments with more physiological models, such as isolated mitochondria and intact cells. The apparent discrepancy between the findings with mitochondria and liposomes suggests that additional molecules may exist in the OMM that are important for this process.

Support for such a notion is presented in a paper by Roucou *et al*.[36] The authors suggest that, although tBid is important for Bax oligomerization, by itself it is not sufficient for membrane permeabilization but requires the presence of an additional, yet unidentified mitochondrial protein. In particular, their findings indicate that oligomerization of Bax occurs neither spontaneously, when monomeric Bax is added to isolated mitochondria, nor when mixtures of monomeric Bax and tBid are added to liposomes consisting of either 30% cardiolipin or lipids isolated from mito-

chondria. Perhaps the most significant finding is that tBid-induced oligomerization of Bax in isolated mitochondria was inhibited when these organelles were pretreated with protease K, an agent used for the general digestion of proteins. Taken together, these findings suggest that an OMM protein, rather than cardiolipin, is required for pore formation and protein efflux induced by mixtures of tBid and monomeric Bax. Several possible targets have been identified, among them the voltage-dependent anion channel in the OMM and the mitochondrial fission machinery.[37,38] However, similarly to cardiolipin, both have been questioned to play an important role in mediating the release of mitochondrial intermembrane space proteins by tBid and Bax.[36,39] To identify such a factor, and to unravel the precise steps of Bax oligomerization, will be an important task for future research.

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Atg5 and Bcl-2 provide novel insights into the interplay between apoptosis and autophagy

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Autophagy and apoptosis play important roles in the development and cellular homeostasis of eukaryotes. Apoptotic cell death is termed type I programmed cell death. Autophagy regulates both cell survival and cell death. While increased numbers of autophagosomes can be associated with cell death (called type II programmed cell death), it is often unclear if this association is causal. Recent data have revealed possible molecular mechanisms for crosstalk between autophagy and apoptosis. Atg5, previously considered to be an autophagy-specific gene involved in autophagosome precursor expansion and completion through an ubiquitin-like conjugation system, now appears to be an important mediator of apoptosis. Atg5 can be cleaved following death stimuli, and the cleavage product appears to promote mitochondria-mediated apoptosis. Bcl-2, the well-characterised apoptosis guard, appears to be important in autophagy, as it binds to

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