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Mitochondrial control of apoptosis: the role of cytochrome *c*

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Abstract

Mitochondrial cytochrome *c* (cyt *c*) has been found to have dual functions in controlling both cellular energetic metabolism and apoptosis. Through interaction with apoptotic protease activating factors (Apaf), cyt *c* can initiate the activation cascade of caspases once it is released into the cytosol. The loss of a component of the mitochondrial electron transport chain also triggers the generation of superoxide. Although cyt *c* can be released independent of the mitochondrial permeability transition (MPT), the accompanying cellular redox change can trigger the MPT. Since another apoptotic protease, AIF, is released by MPT, the two separate pathways provide redundancy that ensures effective execution of the cell death program. Anti-apoptotic Bcl-2 family proteins function as gatekeepers to prevent the release of both cyt *c* and AIF. In spite of their stabilization effect on the mitochondrial outer membrane, Bcl-2 proteins may also be involved in the direct binding of Apaf molecules as regulatory elements further downstream from the mitochondrial apoptotic signals.

Keywords: Apoptosis; Cytochrome *c*; Mitochondrion; Bcl-2; Apaf; Caspase; Membrane potential; Permeability transition; Oxidant-induced cell death; Reactive oxygen species

1. Introduction

In the last 2 years, at least two mitochondrial proteins, cytochrome *c* (cyt *c*) [1] and apoptosis inducing factor (AIF) [2], have been identified as key signaling molecules of apoptosis [3]. These findings have dramatically affected the understanding of the involvement of mitochondria in controlling of cell death. This review will summarize some of these recent findings, particularly relevant to cyt *c*, and discuss possible models for the involvement of mitochondria during apoptosis.

Mitochondria are well known to have a central role in energy metabolism [4], ion homeostasis [5] and redox regulation [6], and damage to the mitochondria has long been linked to cell death [7]. Mitochondrial respiratory inhibitors and agents that induce mitochondrial swelling or loss of the membrane potential can result in cellular energetic failure, Ca^{2+} overloading, generation of reactive oxygen species (ROS), loss of osmotic regulation and cell death. Among numerous examples are KCN toxicity and ischemia, which typically produce a necrotic morphology in which cells swell and lyse, with loss of cytoplasmic contents and appearance of distorted and swollen organelles [8]. Mapping of the mitochondrial genome [9] has also provided the foundation for understanding the role of mitochondria in aging and genetic diseases [10]. However, until recently, the role of mitochondria in cell death was typically consid-

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ered to be passive, reflecting damage to critical mitochondrial functions that would result in cell death.

When Kerr et al. [11] proposed apoptosis as a unique type of cell death that is distinct from necrosis, they pointed out that the ultrastructure of cytosolic organelles, including mitochondria, was well preserved while the nuclear chromatin had aggregated and cytoplasmic contents had condensed as an overall shrinkage of the cell took place [11–13]. Electron microscopy showed that even in phagocytosed apoptotic bodies, mitochondria had nearly normal appearance [11,12]. These morphological findings have been further confirmed by later studies [14,15] and the preservation of mitochondrial structure and function has been viewed as a hallmark of apoptosis, as in contrast to necrosis where mitochondria are swollen and disrupted. Indeed, a normal supply of ATP, as provided by intact mitochondria, would appear as a requisite for 'controlled' cell deletion [16–18]. In support of this, morphologically intact mitochondria were found to be a required component for the nuclear apoptosis in a cell-free system using *Xenopus* egg extracts [19].

However, the early recognition that Bcl-2, a mammalian homologue of the anti-death protein Ced-9 of *Caenorhabditis elegans*, is largely localized on the outer membrane of mitochondria [20–23] forced consideration that the mitochondria may have a role other than simply energy production. An attractive hypothesis was proposed that ROS generated from the mitochondrial electron transport chain could provide an apoptotic signal and Bcl-2 could function as an antioxidant [23,24]. Indeed, over-expression of Bcl-2 was found to inhibit both the nuclear apoptosis and mitochondrial generation of ROS [23,24]. But since cells lacking mtDNA (ρ^0 cells), and cells cultured under anaerobic conditions where the generation of ROS was limited, still could be induced to undergo apoptosis [25–27], this oxidant signaling hypothesis did not provide a totally satisfactory explanation for the role of mitochondria in apoptosis.

2. Mitochondrial cytochrome *c* release and signaling of apoptosis

Considerable clarification for the role of mitochondria in apoptosis was obtained when Liu et al. [1] found that cyt *c* is an essential component of the complex that activates the death protease caspase-3 (CPP32). During purification of the components for the in vitro reconstitution of caspase-3 activation, they found that an active fraction was 'noticeably pink' [1] and had the typical cyt *c* absorbance spectrum. They showed by microsequencing that this was cyt *c*. In non-apoptotic HeLa cell cytosolic extracts, cyt *c*, together with either dATP (μM) or ATP (mM), could activate caspase-3 and subsequent nuclear DNA fragmentation. Immunodepletion of cyt *c* abolished this activation, and addition of purified cyt *c* restored the activation. This research provided the first direct evidence for the involvement of a mitochondrial protein in the activation of apoptosis.

Cytochrome *c* is a 12.3 kDa, nuclear DNA encoded protein. Its precursor, apocytochrome *c*, is synthesized on free ribosomes in the cytoplasm and can spontaneously insert into the mitochondrial outer membrane via a non-receptor mediated process [28,29]. With its further interaction with mitochondrial cytochrome *c* heme lyase, heme is incorporated, and the protein refolds and is released into the mitochondrial intermembrane space. The functional cyt *c* then binds with cytochrome oxidase via its surface positive charges. Both the X-ray crystallographic and solution structures of cyt *c* have been resolved [30–33]. As part of the mitochondrial electron transport chain, cyt *c* has a very well defined and specific function in transfer of electrons between complex III (ubiquinol: cytochrome *c* oxidoreductase) and complex IV (cytochrome oxidase). Thus, the observation that it is a component of the activation system for caspase-3 established a second and apparently independent function of this hemoprotein. Of interest, this latter function does not seem to depend upon the redox activity of the cytochrome [34,35].

Numerous studies with intact cells and in vitro cell free systems have already provided strong support for the findings of Liu et al. [1]. In HL60, CEM, HeLa, Jurkat, U937, FL5.12 and cultured cerebellar granule neuronal cells [36–41], a variety of apoptotic stimuli, including treatment with staurosporine, etoposide, paclitaxel, UVB, ionizing irradiation, anti-Fas, growth factor withdraw, peroxides and MPP^+, all could induce the release of cyt *c* before the caspase-3 activation and nuclear apoptosis (Table 1).

Table 1
Release of mitochondrial cytochrome *c* in apoptosis

Cell type	Inducing agents	Reference
HL 60	Staurosporine Etoposide Ara-C Paclitaxel	[36,39]
CEM	Staurosporine Etoposide Actinomycin D Hydrogen peroxide UVB	[37]
U-937	Ionizing radiation Cisplatinum	[38]
Cerebellar granule neurons (CGN)	MPP^+	[40]
Jurkat	Anti-Fas Staurosporine	[41]
FL 5.12	IL-3 withdrawal	[41]

With in vitro systems, cyt *c* was found to activate caspase in cytosolic extracts isolated from *Xenopus* eggs and Jurkat, HeLa, HL60, CSM 14.1 and NSC cells [34–41]. Where studied, mitochondria were found to maintain the membrane potential while cyt *c* was released and caspases were activated [36,37,41].

Studies of cyt *c* from different species indicate that the role of cyt *c* in apoptosis is widespread and common. Cyt *c* is a highly conserved protein and cyt *c* from horse, bovine, rat, pigeon and tuna [1,34] all could reconstitute the caspase activation in vitro. Yeast cyt *c*, partially acetylated cyt *c* or biotinylated cyt *c* were inactive, as were apocytochrome *c*, heme alone, microperoxidase-11, cytochrome b_5, cytochrome *P*-450 (2E1) or heat inactivated cyt *c* [34,35]. In contrast, the redox state of cyt *c* was not related to the caspase-3 activation, as illustrated by the fact that copper or zinc substituted non-oxidizable cyt *c* were still active. Although potassium ferricyanide could inhibit the caspase-3 activation, this effect was thought to be further downstream from cyt *c* [34,35]. Thus, cyt *c* appears to function as a molecular adaptor rather than as a redox active component during the activation of caspase-3.

Although most studies indicate that cyt *c* loss from mitochondria is due to its release into the cytoplasm, Babior, Gottlieb and co-workers [18,42] suggest that cyt *c* is inactivated during Fas-activated apoptosis in Jurkat cells. By measuring substrate-stimulated respiration rate and using absorption spectroscopy to measure the cytochrome contents, they showed that the measured cyt *c* in both the mitochondrial and post-mitochondrial fractions were not significantly changed during apoptosis, but the electron transfer capacity of cyt *c* in the mitochondrial chain was lost. The inactivation was caused by the permeabilization of the mitochondrial outer membrane, and could be prevented by over-expression of Bcl-2 or by the general caspase inhibitor z-VAD. Because of the inhibition by z-VAD, it appears likely that this inactivation of cyt *c* occurred as a consequence of the receptor-mediated activation of caspase-8 or caspase-1 [43]. While it is possible that their spectral measurements did not have sufficient sensitivity to measure a release of mitochondrial cyt *c* in cultured cells, it should also be pointed out that the Western blot analyses by other investigators do not show whether cyt *c* remains redox active. Thus, it appears possible that the redox activity of cyt *c* is lost in concert with its release, and that inhibition of the redox activity of cyt *c* has an undetermined function during apoptosis.

3. Mechanism of cytochrome *c*-dependent activation of caspase-3

The mechanism of cyt *c*-dependent activation of caspase-3 has been recently elucidated by Wang and co-workers [44,45]. They purified Apaf-1 (apoptotic protease activating factor-1) [44] and found it to be the first identified mammalian homologue of Ced-4 that couples cyt *c* to the activation of caspase-9. The N terminus of Apaf-1 has the CARD domain (caspase recruitment domain [46]), which is shared by caspases 1, 2, 3, 4, 9 and Ced-4. The C-terminal side after the CARD domain is the Ced-4 homologue region, which contains Walker's A- and B-box consensus sequences. The rest of the C terminus of Apaf-1 has 12 WD-40 repeats.

Activation is thought to require two steps, binding of ATP or dATP to Walker's boxes, and binding of

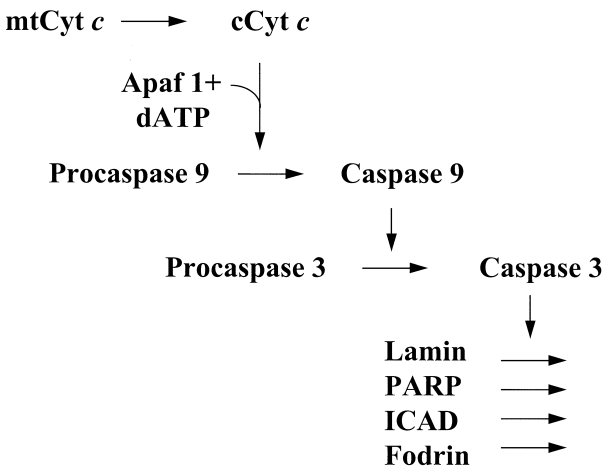

Fig. 1. Activation cascade initiated by mitochondrial cytochrome *c* release. Release of mitochondrial cytochrome *c* (mtCyt *c*) from the intermembrane space into the cytoplasm (cCyt *c*) allows interaction with Apaf-1 which, in the presence of micromolar concentration of dATP or millimolar concentration of ATP, results in proteolytic activation of procaspase-9. Active caspase-9 activates procaspase-3 by proteolytic cleavage. Active caspase-3 subsequently cleaves a variety of substrates resulting in characteristic morphologic changes in the nucleus, DNA fragmentation, and appearance of the phagocytic marker phosphatidylserine on the cell surface. Multiple signaling events may activate cyt *c* release, but these have not yet been clearly defined. Bcl-2 functions in regulating cyt *c* release, but probably has additional functions. Proximal aspects of the mitochondrial activation pathway are distinct from those of the Fas-activation pathway but the two pathways converge at distal steps with activation of caspase-3.

cyt *c* to the WD repeats. These interactions allow a conformational change that exposes the CARD domain, where caspase-9 (Apaf-3) can bind and be activated (Fig. 1). Since Apaf-1 does not have caspase activity, caspase-9 is possibly cleaved through autocatalysis. Active caspase-9 cleaves and activates caspase-3 in a cascade that subsequently cleaves caspase-6, DFF (DNA fragmentation factor) [47], PARP (poly(ADP-ribose)polymerase) and other substrates, leading to the nuclear lamin cleavage, DNA fragmentation, and other characteristic changes of apoptosis [48]. Thus, the release of cyt *c* from the mitochondria provides a key signal that initiates the irreversible death sequence and brings the affected cells to the no-return point.

While cyt *c* release is a proximal signal in the activation of caspase-3 by this sequence, it has also become apparent that cyt *c* release is distal to caspase activation in Fas-activated apoptosis. With this mechanism of activation of apoptosis, mitochondrial signaling seems to be unnecessary, but may nonetheless contribute to apoptosis as an amplification mechanism. The death effector domain (DED) [49] of the Fas receptor associated protein FADD/MORT1 [50,51] has structural homology to the CARD domain and the death domain (DD) of Fas receptor [46]. DED is shared by FADD/MORT1, caspase-8 and TRADD (TNF receptor associated protein) [52]. Like CARD, DED can be used by FADD to recruit and activate caspase-8 [53,54], in a process that occurs within minutes after receptor oligomerization. The activated caspase-8 cleaves and activates caspase-1, and the subsequent apoptotic cascade.

Mitochondrial cyt *c* loss occurs as a consequence of this upstream caspase activation, and general caspase inhibitors such as z-VAD block cyt *c* loss [15,18]. Thus, in contrast to the system described by Wang and colleagues, mitochondria apparently have a distal role in Fas-activated apoptosis. Similarly, with TNF receptor mediated apoptosis, where either TRADD recruits FADD/caspase-8 complex by interaction of the DED domains or RAIDD recruits caspase directly via their CARD domains [52,55,56], mitochondria appear to have a downstream signaling role. Discrimination between these pathways and those dependent upon cyt *c* release provides an important foundation for the future classification of apoptotic signaling pathways.

4. Gatekeeper function of Bcl-2 family proteins

Bcl-2 was originally recognized as an inhibitor of apoptosis and found to be homologous to Ced-9, the antiapoptotic protein of *C. elegans*. [57]. More than ten members of the Bcl-2 family have been identified and some of these have pro-apoptotic instead of anti-apoptotic activity [58]. Despite intense investigations, the mechanism of action of the Bcl-2 family proteins in apoptosis has remained elusive, and this has recently been further confused by the recognition of functions of these proteins that are apparently not related to apoptosis. These other functions include a role in the regeneration of neurons [59] and transcriptional regulation through the interaction with NF-AT and calcineurin [60]. Thus, even though

Bcl-2 family proteins are mammalian homologues of Ced-9, their roles in mammalian cell function may be quite different from that of Ced-9 in *C. elegans*.

The observation that over-expression of Bcl-2 and Bcl-X_L prevented the release of cyt *c* into the cytosol provided an important insight into the possible anti-apoptotic mechanisms [36–39,41]. Cyt *c* is on the outside of the inner mitochondrial membrane while Bcl-2 is associated with the outer membrane, extending to the inner surface; how they interact is not clear. Bcl-2 or Bcl-X_L over-expression protects against apoptosis in ρ^0 cells which do not have functional mitochondria [25–27] and also in cells micro-injected with cyt *c* [61,62]. These observations, plus the recognition that Bcl-2 has functions unrelated to apoptosis, suggest a range of possible mechanisms of action for Bcl-2 family members (Fig. 2). These include functions in channel formation and ion movement, action as a chaperone or molecular gatekeeper that controls movement of proteins such as cyt *c* across membranes, binding to apoptosis proteins as an adaptor or docking element and as a membrane stabilizing agent that preserves integrity of membranes.

The X-ray crystallographic structure suggested a pore-forming capacity of Bcl-2 family proteins [63]. The arranged two central hydrophobic α-helices surrounded by five amphipathic α-helices structure of Bcl-X_L resembles that of bacteria diphtheria toxin [63], and provides a theoretical structural base for membrane insertion and pore forming function. In synthetic lipid membranes, pro-apoptotic members, such as Bax, can form anion selective channels, while anti-apoptotic one, Bcl-2 and Bcl-X_L, form cation selective channels [64–67]. Because the whole length proteins are highly insoluble, all these studies used C-terminal truncated proteins. The binding and insertion of truncated proteins, especially anti-apoptotic ones, are pH dependent. The transmembrane ion flow could be increased by acidifying the solution to pH 4.0, and at pH 6.0, the current through Bcl-X_L and Bcl-2 was not detectable. Interestingly, although both Bcl-2 and Bax form channels in synthetic lipid membranes, only Bax could lyse the neurons or erythrocytes when added extracellularly. One explanation could be that channels formed by the anti-apoptotic ones remain closed while ones formed by Bax are 'constitutively active'. By heterodimerization on the mitochondrial outer membrane, these proteins can either promote or inhibit the cyt *c* release, depending on their levels of expression, and perhaps more importantly, the ratio between them. However, over-expression of Bcl-X_L mutants that do not bind with Bax can still inhibit apoptosis [68] or Bax-induced cell death in budding yeast [69]. Over-expression of Bcl-2 inhibited apoptosis induced by direct injection of cyt *c* [61]. Thus, even if the Bcl-2 family members do have a channel-forming character, it is difficult to envisage how these channels function to promote or inhibit apoptosis. A further downstream effect after the mitochondria is likely to be involved.

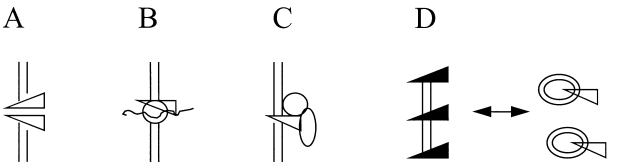

Fig. 2. Functions of Bcl-2 family proteins. Bcl-2 was originally identified as an anti-apoptotic protein but is now recognized to be a member of a larger family of proteins with pro-apoptotic as well as anti-apoptotic members. The emerging view is that the opposing functions are regulated transcriptionally as well as by phosphorylation/dephosphorylation, proteolysis and protein-protein interactions. Suggested functions include: A, as a channel; B, as a chaperone or gatekeeper that controls chaperone function; C, as an adaptor or docking protein; D, as a protein that regulates membrane stability. As a channel (A) pro-apoptotic members have an anionic specificity and anti-apoptotic members have a cationic specificity. As a chaperone or gatekeeper of chaperone activity (B), pro-apoptotic members promote movement in a death-signaling direction (e.g., cyt *c* release, loss of protease compartmentation) while anti-apoptotic members block such movement. As an adaptor or docking protein (C), pro-apoptotic members could facilitate activation of apoptosis by bringing key elements together while anti-apoptotic members could serve to block such interactions. As a protein that stabilizes membranes (D), the activity can be viewed in analogy to the components that function in fragmentation of the nuclear envelope during mitosis. Pro-apoptotic members will destabilize the membrane, promoting release of intermembrane components (i.e., cyt *c* from mitochondria) or fragmentation into apoptotic bodies (i.e., nuclear and plasma membrane blebbing). Anti-apoptotic members will function as membrane stabilizing or vesicle fusion proteins.

The conformational flexibility of apocytochrome *c* that allows it to spontaneously insert and move through the mitochondrial outer membrane is lost upon insertion of the heme [28,29]. Thus, an alternative function for Bcl-2 family members related to

channel-forming ability could be to function as a chaperone molecule, or a molecular gatekeeper that works in concert with a chaperone system, to control movement of macromolecules from one compartment to another. The research of Samali and Cotter [70] suggests involvement of chaperones in control of apoptosis because increased expression of heat shock proteins inhibit apoptosis. Moreover, BAG-1, a multifunctional protein that binds Bcl-2, regulates chaperone activity of Hsc70 and also inhibits apoptosis [71]. Such a gatekeeper activity of Bcl-2 family members would place these proteins functionally at a position where they could control processes such as release of mitochondrial cyt *c* to activate apoptosis and also have other functions such as in the nuclear uptake of transcription factors or the movement of proteins across the endoplasmic reticulum. Regulation of Bcl-2 via phosphorylation, proteolysis or interaction with other family members could transform it from an inhibitor to a mediator of transmembranal protein trafficking. Such an activity could also explain the rather enigmatic localization of Bcl-2 in different membrane compartments and the ability of Bax and other family members to induce cell death.

On the other hand, Bcl-2 family members directly bind with apoptosis elements in vitro [38], suggesting that Bcl-2 family members could have direct protein regulatory activity, perhaps being an adaptor or docking protein [72]. This was determined with co-immunoprecipitation, Far Western blot and ELISA. Bcl-Xs did not bind cyt *c*, and excess Bcl-Xs could compete off the binding. Although this does not exclude a channel or gatekeeper mechanism, it provides an alternative mechanism to inhibit the cyt *c*-dependent caspase activation. Moreover, it implies a further downstream effect of Bcl-2 family proteins after the cyt *c* is released or when cyt *c* is microinjected into cells [61].

More recently, Bcl-X_L has been shown to protect against mitochondrial swelling and outer membrane breakdown [41]. Anti-Fas, staurosporine and IL-3 withdrawal all induced an early hyperpolarization and swelling of mitochondria before cyt *c* was released. Both the volume and potential change contributed to the increased mitochondrial uptake of potential-dependent fluorescent dye during early apoptosis, a phenomenon which had been reported before [73]. Inconsistent with other studies of mitochondria during apoptosis, high resolution electron microscopy showed a disruption of the mitochondria outer membrane, which the authors concluded was due to the initial swelling of mitochondria during early stages of apoptosis. Thus, they interpreted that cyt *c* was released once the outer membrane was damaged. Further support came from the fact that over-expression of Bcl-X_L prevented oligomycin induced hyperpolarization of mitochondria and subsequent mitochondrial swelling and cytochrome *c* release. Similar results were obtained with antimycin A, which also caused mitochondrial swelling and cytochrome *c* release that were blocked by over-expression of Bcl-X_L. Thus, an alternate function of Bcl-2 family members, as illustrated by this Bcl-X_L study, is that they could act to protect the integrity of the mitochondrial outer membrane regardless of the nature of initial stimulus.

While such a possibility may seem somewhat far-fetched, it should be noted that Bcl-2 over-expression has been previously reported to protect against necrotic cell death [74], suggesting that membranal stabilization may be an important function of this protein. As membrane budding and fusion are ongoing, controlled processes in cells and the nuclear envelope undergoes a controlled fragmentation and reformation during mitosis, a role for Bcl-2 family members in membrane stability is rather appealing because it suggests functions of Bcl-2 family members that could be important in normal cell growth and differentiation, as well as apoptosis. Pro-apoptotic members could facilitate membrane blebbing and fragmentation while anti-apoptotic members could stabilize large continuous membrane domains.

5. Is caspase activation synonymous with apoptosis?

In earlier studies of apoptosis, DNA fragmentation was taken as a 'biochemical hallmark of apoptosis' [75]. Subsequent studies revealed that DNA fragmentation was not a ubiquitous characteristic of cells with morphologic features of apoptosis [76] and only recently has an appropriate endonuclease system been identified [77]. Thus, it is appropriate to raise the question whether sequential activation of caspases provides a useful biochemical definition

of apoptosis, or whether the definition of apoptosis should be maintained strictly as a morphological term.

The question needs to be addressed in three ways: whether the spectrum of caspases and morphologic changes associated with their activation are suitably similar to warrant inclusion within a single conceptual framework, whether some of the caspases have functions other than apoptosis, and whether morphologic processes currently identified as apoptosis can occur via activation processes not involving caspases. Considerable information is available on the caspases and distinction between activation sequences, such as between Fas-activated and cyt *c*-mediated pathways are already apparent. Thus, it appears likely that further classification of apoptosis will be possible based upon morphologic and biochemical characteristics, and, within this framework, that mitochondria-dependent mechanisms will constitute at least one of these classes.

However, simple definition of apoptosis in terms of caspase-dependent signaling will probably be elusive even though these important enzymes may be central to apoptosis in most systems. Caspase-1, interleukin-1β converting enzyme (ICE), was known before the family of 'death proteases' was recognized [78]. Non-apoptotic functions of caspases are likely to exist and are being discovered. For instance, caspase-3 has recently been identified to be required for the processing and activation of pro-interleukin-16 [79]. Pro-interleukin-16 has no significant sequence similarity to pro-interleukin-1β, and the spectra of biological activities of these interleukins are different [79]. Thus, the results indicate that even caspase-3, the protease most closely homologous with the *C. elegans* death protease Ced-3 and playing the critical role in cyt *c*-dependent apoptosis, may have non-apoptotic functions.

Further investigation is likely to reveal activation systems for controlled cell deletion that are signaled by caspase-independent mechanisms. For instance, activation of the respiratory burst in neutrophils with phorbol ester is followed by cell death that has neither apoptotic nor necrotic morphology [80], and so do the L929 fibroblast cells treated with 0.1 and 0.5 mM H_2O_2 for up to 2 days [81]. Whether such cell death is dependent upon caspases is not yet apparent, but such cases will require that either 'apoptosis' be redefined in terms of the biochemistry or that more critical morphologic criteria be established. In either case, additional efforts are needed to allow discrimination of caspase-dependent and caspase-independent cell death and ultimately determine whether caspase activation provides a useful biochemical definition that is synonymous with the morphologic definition of apoptosis.

6. Redox signaling of apoptosis

An unresolved issue of mitochondria-mediated apoptosis lies in the role of redox signaling. Considerable circumstantial evidence suggests that mitochondrial signaling of apoptosis may not be limited to the role of cyt *c* in activation of caspase-3 and that reactive oxygen species (ROS) may provide an alternate signaling pathway. Several studies show that Fas activation and TNFα treatment of cells results in stimulated mitochondrial generation of ROS [82]. Moreover, a variety of oxidants activate apoptosis [83], induction of apoptosis is commonly associated with an oxidation of the cellular glutathione pool [84], and thiols such as *N*-acetylcysteine commonly inhibit apoptosis [85]. Because mitochondria have a central role in cellular redox regulation, these separate observations indicate that mitochondria may provide a redox signal to activate the key processes of apoptosis in parallel to the cyt *c*-dependent activation of caspase-3.

It is well known that the mitochondrial permeability transition (MPT) is activated by oxidants [86], and the data by Kroemer and colleagues show that the MPT is associated with apoptosis in many systems [87]. Thus, the alteration in mitochondrial electron transport and oxidant production associated with loss of cyt *c* could activate the MPT and provide an alternate apoptosis signaling mechanism that is functionally redundant to the caspase-3 activation and serves to guarantee cell death.

MPT is a well studied phenomenon [86] wherein the normally highly impermeable mitochondrial inner membrane abruptly becomes permeable to molecules of less than 2 kDa. The MPT occurs as a consequence of the reversible opening of megachannels at the contact regions on the inner and outer membranes of mitochondria. Upon opening of the

permeability pore, rapid ion movement causes extensive mitochondrial swelling and loss of the mitochondrial membrane potential. Kroemer and his co-workers have postulated that the MPT is a common apoptotic 'effector'. A protein factor of around 50 kDa, AIF, is suspected to be released from mitochondria following the MPT and function as a central executioner of apoptosis [2]. A review on this topic is included in the present issue of this series [88].

Although isolated mitochondria can release cyt *c* under in vitro conditions when MPT is triggered [89], in the context of an intact cell, cyt *c* can be released when most of the mitochondria retain a normal or near-normal membrane potential. This has been reported by separate studies with HL60 cells, Jurkat cells, PC-12 cells, FL5.12 pro-B, using different apoptosis inducing agents [36,37,41]. Although it remains possible that a small subfraction of mitochondria can undergo the MPT, release cyt *c* or AIF and induce apoptosis, it is clear that most of the mitochondria do not undergo the permeability transition early enough to explain cyt *c* release under these conditions. Furthermore, microinjection of cyt *c* into different cell types induces apoptosis, apparently without activation of the MPT [61,62]. Thus although MPT is sufficient to release cyt *c*, it is not required for its release.

The study of Krippner et al. [15] showed that mitochondrial respiration is dramatically inhibited as a consequence of loss of cyt *c* activity in Fas-activated Jurkat cells. We have found that induction of apoptosis in HL60 cells with TNFα or staurosporine similarly resulted in inhibited mitochondrial respiration [90]. Inhibition of normal electron transport through the mitochondrial electron transport chain with cyanide or antimycin A results in diversion of electron flow from the middle portion of the chain directly to O_2 to produce superoxide [91]. Similarly, loss of cyt *c* from mitochondria, as occurs during apoptosis, results in stimulated superoxide production [90]. Prevention of cyt *c* release from mitochondria by overexpression of Bcl-2 also prevented the increase in superoxide production. Thus, enhanced mitochondrial production of reactive oxygen species appears to be a consequence of cyt *c* release, and the antioxidant function of Bcl-2 is due to its effect on cyt *c* release. This, together with the export of reduced glutathione from apoptotic cells [92], can lead to dramatic oxidation of intracellular thiol pools. Since MPT pore has critical thiol groups, the redox change subsequent to the cyt *c* release can trigger the MPT.

MPT can also be activated by caspases such as occurs in Fas-mediated apoptosis, where it is blocked by the general caspase inhibitor, z-VAD [93]. Taken together, a picture emerges that MPT and cyt *c* release are two separate but interacting events. MPT can release cyt *c*, and vice versa. The final execution phase of apoptosis does not appear to need the activation of both pathways. Rather, this apparent redundancy may provide a fail-safe system that insures cell death even when one of the pathways does not function properly. Once activated, either pathway can execute a death program and together they provide a positive feedforward mechanism that protects against failure of apoptosis.

7. Mitochondria-mediated apoptosis in disease

The importance of apoptosis in human disease and its treatment has been widely recognized and frequently reviewed [94]. In cardiac ischemia-reperfusion, mitochondria are central to the ROS generation and calcium-associated damage [95]. In numerous types of chemical-induced liver or renal injury, toxic effects are thought to occur via effects on the mitochondria [96]. Oxidative damage to mitochondria also appears to have a central role in neurodegenerative diseases [97]. Defective apoptosis contributes to malignancy, and subnormal and aberrant mitochondria may contribute to the resistance of tumor cells to apoptosis [98]. Thus, the recognition of mitochondrial signaling provides an important opportunity for improved understanding of the role of apoptosis in these disease processes.

While review of all these diverse pathologic processes is beyond the scope of this brief review, two general aspects are worthy of consideration, namely that mitochondrial signaling of apoptosis evolved to protect against the age-related oxidative damage to mitochondria and that model systems used to study apoptosis may be largely inadequate to investigate age-dependent diseases of primary interest.

The mitochondrial genome is more sensitive to mutation than the nuclear genome, and mtDNA

damage, including both base damage and large deletions, has been found to increase with age in all post-mitotic tissues studied so far [99,100]. Mitochondrial DNA is not covered by histones. It is localized near a major intracellular source of ROS and, compared to the nuclear DNA, has a high transcription rate. The DNA repair system inside the mitochondria is not efficient. After the same level of oxidative damage, mtDNA damage remains much longer than that of nuclear DNA [101]. Thus, as age increases, mtDNA damage increases and is possibly associated with decreased mitochondrial functions and increased generation of ROS. Apoptosis could be induced by either a release of cyt *c*, by ROS signaling or by the MPT-induced release of AIF. This would, on the one hand, protect against oxidative mutations in the nuclear genome and therefore prevent malignant transformation. On the other hand, it would also cause the loss of functional cells and potentially could contribute to degenerative processes. The former may be particularly important in epithelial cells exposed commonly to oxidants and other toxic chemical species while the latter would be particularly important for tissues such as the central nervous system and the retina.

Unfortunately, it is not apparent that the currently available models for study of apoptosis are suitable for providing a clear understanding of the role of apoptosis in these relevant disease models. Apoptosis is often studied with hematopoietic cells that normally have a very short half life and also have specialized systems for apoptosis related to immune functions. This problem is compounded by the fact that most are derived from malignancies that have aberrant apoptotic systems. A further problem is that conditions are usually used which provide a substantial rate of apoptosis. In contrast, a very low frequency of cells is affected in most relevant age-related disease processes. For instance, a change in frequency of apoptosis from 0.01% per day to 0.02% per day may mean that an individual develops macular degeneration at age 60 instead of age 90 [102]. Such a low frequency of cell death will be hard to discriminate from the spontaneous cell death under in vitro cultured conditions. At present, we do not know whether acute toxicity model systems in which more than 50% of the cells undergo apoptosis in 4–12 h are relevant to apoptosis as it occurs during aging.

8. Conclusions

While there remain considerable questions concerning the role of mitochondria-induced apoptosis in human disease, recent advances in understanding mechanisms whereby mitochondria can activate apoptosis provide a substantial improvement in knowledge of this important process. It is now clear that a central and common pathway for signaling apoptosis involves release of cyt *c* from mitochondria and subsequent activation of the death protease, caspase-3. This process is regulated in an unknown way by members of the Bcl-2 family proteins. A mitochondrially derived redox signal is simultaneously generated as a consequence of mitochondrial cyt *c* release. This, together with the MPT, may function as an alternate 'fail-safe' signaling pathway for apoptosis or function in activation of specific processes of the execution program. However, non-mitochondrial signaling pathways for activation of caspases and induction of apoptosis are known and therefore additional studies are needed to provide a suitable delineation of the role of mitochondria-mediated apoptosis in the broader spectrum of apoptotic signaling mechanisms.

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