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THE MITOCHONDRIAL DEATH/LIFE REGULATOR IN APOPTOSIS AND NECROSIS

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ABSTRACT

Both physiological cell death (apoptosis) and, in some cases, accidental cell death (necrosis) involve a two-step process. At a first level, numerous physiological and some pathological stimuli trigger an increase in mitochondrial membrane permeability. The mitochondria release apoptogenic factors through the outer membrane and dissipate the electrochemical gradient of the inner membrane. Mitochondrial permeability transition (PT) involves a dynamic multiprotein complex formed in the contact site between the inner and outer mitochondrial membranes. The PT complex can function as a sensor for stress and damage, as well as for certain signals connected to receptors. Inhibition of PT by pharmacological intervention on mitochondrial structures or mitochondrial expression of the apoptosis-inhibitory oncoprotein Bcl-2 prevents cell death, suggesting that PT is a rate-limiting event of the death process. At a second level, the consequences of mitochondrial dysfunction (collapse of the mitochondrial inner transmembrane potential, uncoupling of the respiratory chain, hyperproduction of superoxide anions, disruption of mitochondrial biogenesis, outflow of matrix calcium and glutathione, and release of soluble intermembrane proteins) entails a bioenergetic catastrophe culminating in the disruption of plasma membrane integrity (necrosis) and/or the activation of specific apoptogenic proteases (caspases) by mitochondrial proteins that leak into the cytosol (cytochrome *c*, apoptosis-inducing factor) with secondary endonuclease activation (apoptosis). The relative rate of these two processes (bioenergetic catastrophe versus protease and endonuclease activation) determines whether a cell will undergo primary necrosis or apoptosis. The

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acquisition of the biochemical and ultrastructural features of apoptosis critically relies on the liberation of apoptogenic proteases or protease activators from mitochondria. The fact that mitochondrial events control cell death has major implications for the development of cytoprotective and cytotoxic drugs.

APOPTOSIS AND NECROSIS—DIFFERENCES BETWEEN TWO MODES OF CELL DEATH

Cell death constitutes one of the key events in biology. At least two modes of cell death can be distinguished: apoptosis and necrosis. Apoptosis is a strictly regulated (programmed) device responsible for the ordered removal of superfluous, aged, or damaged cells (1, 2). Every second, several millions of cells of the human body undergo apoptosis; i.e. in conditions of homeostasis, each mitosis is compensated by one event of apoptosis. It is likely that all cells of the human body possess the intrinsic capacity of undergoing apoptosis, even in the absence of de novo protein synthesis (3), which suggests that all structures and processes required for at least one pathway to apoptosis are ubiquitously present (and probably necessary for cell survival). Of course, macromolecular synthesis may be required for certain agents to cause apoptosis—either because they have differing pathways or because of linkages to the preexisting proteins particular to these agents. Disturbances in apoptosis regulation illustrate the importance of apoptosis for normal homeostasis. An abnormal resistance to apoptosis induction correlates with malformations, autoimmune diseases, or cancer due to the persistence of superfluous, self-specific immunocytes, or mutated cells, respectively. In contrast, enhanced apoptotic decay of cells participates in acute pathologies (infection by toxin-producing microorganisms, ischemia-reperfusion damage, or infarction) as well as in chronic diseases (neurodegenerative and neuromuscular diseases, AIDS). Although apoptosis is necessary for both health and disease, necrosis is always the outcome of severe and acute injury: i.e. abrupt anoxia, sudden shortage of nutrients such as glucose, or extreme physicochemical injury (heat, detergents, strong bases etc).

In contrast to necrosis, apoptosis involves the regulated action of catabolic enzymes (proteases and nucleases) within the limits of a near-to-intact plasma membrane (1, 2). Apoptosis is commonly accompanied by a characteristic change of nuclear morphology (chromatin condensation, pyknosis, karyorrhexis) and of chromatin biochemistry (step-wise DNA fragmentation culminating in the formation of mono- and/or oligomers of 200 base pairs). It also involves the activation of specific cysteine proteases (caspases) that cleave after

aspartic acid residues. Caspases catalyze a highly selective pattern of protein degradation (4, 5). Subtle changes in the plasma membrane occur before it ruptures. Thus the surface exposure of phosphatidylserine residues (normally on the inner membrane leaflet) allows for the recognition and elimination of apoptotic cells by their healthy neighbors, before the membrane breaks up and cytosol or organelles spill into the intercellular space and elicit inflammatory reactions (6). Moreover, cells undergoing apoptosis tend to shrink while reducing the intracellular potassium level (7). During the process of apoptosis, mitochondria do not manifest any major ultrastructural abnormalities (8, 9).

In contrast to apoptosis, necrosis does not involve any regular DNA and protein degradation pattern and is accompanied by swelling of the entire cytoplasm (oncosis) and of the mitochondrial matrix, which occur shortly before the cell membrane ruptures (10). The fundamental differences between apoptosis and necrosis are summarized in Table 1.

The morphological features of both modes of cell death—normal or condensed mitochondria in apoptosis and swollen mitochondria in necrosis—have given rise to the erroneous speculation that mitochondria are major players in necrosis but irrelevant to apoptosis. Indeed, the volume of deenergized mitochondria is determined by the cytosolic colloidosmotic pressure (which is

Table 1 Apoptosis versus necrosis: a comparison

Apoptosis	Primary necrosis
Physiological or pathological (subnecrotic damage)	Accidental
Susceptibility tightly regulated	Always pathological
Plasma membranes near-to-intact until late	Unregulated or poorly regulated
Heterophagic elimination	Plasma membrane destroyed early
No leakage of cell content; little or no Inflammation	Leakage of cell contact inflammation
Cellular enzymes participate, causing characteristic biochemical or morphological features including chromatin condensation (pyknosis), nuclear fragmentation (karyorrhexis), regular DNA fragmentation pattern (endonucleolysis), selective protein degradation by specific proteases (caspases), subtle changes in plasma membranes (loss of membrane asymmetry before loss of membrane integrity), cell shrinkage, no mitochondrial swelling	Biochemical and morphological features include swelling of the entire cytoplasma (oncosis), mitochondrial swelling
	Secondary necrosis
	Cytolysis secondary to apoptosis when dying cells fail to be removed by heterophagy

maintained in apoptosis and reduced in necrosis) rather than by mitochondrion-intrinsic parameters. Functional analyses conducted during the past few years have revealed that changes in mitochondrial membrane are as critical for the apoptotic process as they are for necrosis. The present review focuses on the role of mitochondria in death/life decision making.

APOPTOSIS AND NECROSIS—SIMILARITIES AND EARLY INVOLVEMENT OF MITOCHONDRIA

Although apoptosis and necrosis have long been viewed as opposed by antinomy, it is now generally assumed that both forms of cell death constitute two extremes of a continuum (Table 2). Moreover, several observations shed doubt on the functional opposition between these two types of cell death. Thus the same toxin can induce apoptosis or necrosis at a low (subnecrotic) or high dose, respectively (11). The equilibrium between apoptosis and necrosis can be influenced by manipulation of the ATP levels and caspase activation. Thus maintenance of high ATP favors apoptosis over necrosis (12, 13), whereas inhibition of caspase activation transforms apoptosis into necrosis (14–16). As shown in Figure 1, thymocytes normally respond to stimulation

Table 2 A few arguments against the antithesis between apoptosis and necrosis

Argument	Reference
After apoptosis, cells undergo secondary necrosis	(1, 11, 80)
The same toxin can induce apoptosis at a low (subnecrotic) dose and primary necrosis at a high dose	(2, 11)
Many pathologies labeled necrotic involve apoptosis (apoplexy, myocardial infraction, anoxia, ischemia-reperfusion damage, etc)	(17, 18, 81, 82)
Mitochondrial permeability transition is involved in both apoptosis and necrosis	(2, 23)
The oncoprotein Bcl-2 can inhibit both apoptosis and, in some models, primary necrosis	(17, 20–22)
Modulation of cellular ATP levels can shift apoptotic responses to necrosis (low ATP) and vice versa (high ATP)	(12, 13)
Overexpression of Bax and Bak causes apoptosis and, in the presence of caspase inhibitors, non-apoptotic (necrotic?) cytolysis	(14, 15)
Classical apoptosis inducers (serum withdrawl, c-Myc overexpression, etoposide, glucocorticoids) induce non-apoptotic cells death in the presence of caspase inhibitors such as Z-VAD.fmk	(15, 16)

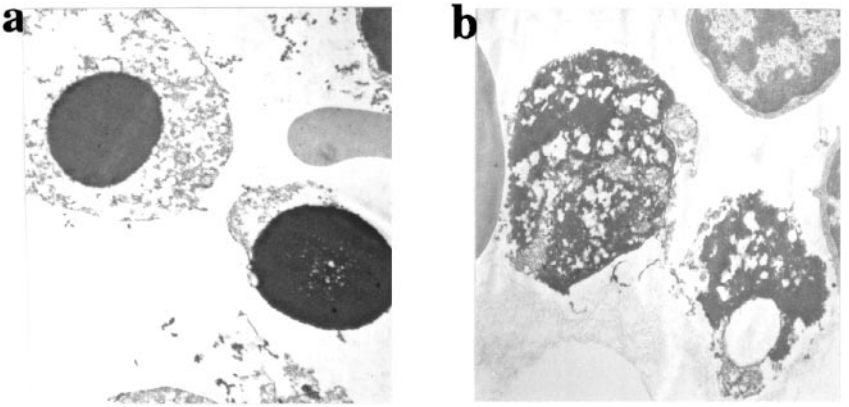

Figure 1 Primary and secondary necrosis. Mouse thymocytes were treated for 12 h with 1 μM dexamethasone, either in the absence (*a*) or in the presence (*b*) of Z-VAD.fmk. Note the typical apoptotic nuclear morphology (condensed, homogeneously electron-dense nuclei) in lysed cells visible in *a*, that is not observed in *b*. The cells have either undergone necrosis after apoptosis (secondary necrosis; *a*) or undergone necrosis in the absence of apoptosis (primary necrosis; *b*).

with glucocorticoids by undergoing apoptosis, followed by secondary necrosis. However, in the presence of the caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD.fmk), cells fail to undergo nuclear apoptosis and die from cytolysis without apoptosis (primary necrosis). A number of pathologies previously thought to involve primary necrosis now appear to involve apoptosis: apoplexy (17), myocardial infarction (18), and ischemia/reperfusion damage (19). More importantly, hyperexpression of the apoptosis-inhibitory oncoprotein Bcl-2 inhibits several examples of necrotic cell death: kainate-induced neuronal necrosis (20), occlusion of the midbrain artery (17), anoxia (21), and chemical hypoxia (22). At least in some instances, mitochondrial permeability transition (PT), which is regulated by Bcl-2, constitutes a molecular event involved in both apoptosis and necrosis (2, 23; Table 3). Thus the early phase of both modes of cell death may involve a similar change in mitochondrial membrane permeability. In the following sections, we discuss the role of mitochondrial changes in necrosis and apoptosis.

MITOCHONDRIAL CHANGES IN NECROSIS: THE PUTATIVE ROLE OF PERMEABILITY TRANSITION

The mitochondrial transmembrane potential ($\Delta\Psi_m$) can be monitored in living cells using a number of different potential-sensitive dyes: rhodamine 123, 3,3′ dihexyloxacarbocyanine iodide [$DiOC_6(3)$], 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1), and chloromethyl-X-

Table 3 Mitochondrial permeability transition in different models of necrosis[a]

Cell type + lethal stimulus	Intervention preventing early $\Delta\Psi_m$ disruption and cell death[b]
Myocardiocytes + oxidative stress	Cyclosporin A (83)
Myocardiocytes + ischemia/reperfusion	Cyclosporin A (84)
Hepatocytes + anoxia/reperfusion	Cyclosporin A or L-carnitine (85)
	Cyclosporin A + BAPTA-AM (intracellular Ca^{2+} chelator) (28)
Hepatocytes + 1-methyl-4-phenylpyridinium	Cyclosporin A (86)
Hepatocytes + protoporphyrin IX	Cyclosporin A (34)
Hepatocytes + *ter*-butylhydroperoxide	Cyclosporin A (27, 87)
	Cyclosporin A plus trifluoperazin (26, 88)
L929 fibrosarcoid cells + tumor necrosis factor α	Cyclosporin A (35)
Hippocampal neurons + glutamate	Cyclosporin A (89)
Cerebellar granule cells + glutamate	Cyclosporin A (90)
Cortical neurons + glutamate	Cyclosporin A (91)
Cortical neurons + *N*-methyl-D-aspartate	Cyclosporin A (92)
PC12 pheochromocytoma cells + cyanide	Bcl-2 or Bcl-X_L overexpression (93)

[a]Note that in several instances the indicated treatment may also induce apoptosis.
[b]References are in parentheses.

rosamine (CMXRos) (24). All these dyes share a cationic lipophilic structure allowing them to distribute freely through membranes into the mitochondrial matrix, as a function of the Nernst equation, correlated to the $\Delta\Psi_m$. With use of these dyes, it has been shown in numerous in vitro models of necrosis that following toxin exposure the $\Delta\Psi_m$ dissipates before the plasma membrane disrupts and before cells manifest signs of damage such as vacuolization or cytoplasmic swelling (Table 3). The $\Delta\Psi_m$ results from the unequal distribution of ions (mainly protons) on the inner mitochondrial membrane. The $\Delta\Psi_m$ disruption suggests that the proton-moving force and/or the inner membrane permeability has been affected during cell damage. In a series of elegant confocal microscopy studies, Lemaster and colleagues (25, 26) showed that when calcein, a polyanionic membrane impermeant fluorescein derivative, is incorporated into cells, all cellular compartments are stained except mitochondria. During hepatocyte necrosis this dye can access the mitochondrion as soon as the $\Delta\Psi_m$ breaks down, indicating that the mitochondrial membranes have indeed become permeant.

Pharmacological experiments have shown that cyclosporin A can prevent or retard the $\Delta\Psi_m$ disruption as well as subsequent cell death (Table 3). In addition, the cyclosporin A derivative *N*-methyl-Val-cyclosporin A, which loses its calcineurin-dependent immunosuppressive effects but conserves its effects on mitochondrial cyclophilin D, remains capable of stabilizing the $\Delta\Psi_m$ and

of exerting cytoprotective effects (Table 3). Based on these observations and additional experiments demonstrating the role of mitochondrial Ca^{2+} fluxes (10, 27, 28), it has been postulated that a cyclosporin A-inhibitable mitochondrial megachannel might be involved in necrosis. This megachannel, also called the permeability transition (PT) pore (29, 30), has been subject to extensive pharmacological and functional characterization (see below).

Depending on different physiological effectors, the PT pore complex can adopt an open or closed conformation. In normal circumstances, most if not all PT pores are closed (29, 30). Opening of the pore has dramatic consequences on mitochondrial physiology, including $\Delta\Psi_m$ collapse, uncoupling of the respiratory chain, and efflux of small molecules and some proteins from the mitochondrion (29, 30). In addition to cyclophilin D, the target of cyclosporin A and *N*-methyl-Val-cyclosporin A, other proteins such as the peripheral benzodiazepin receptor (PBR) and the adenine nucleotide translocator (ANT) have been implicated in the formation and/or regulation of the PT pore (29–33). Substances that specifically act on mitochondrial structures to induce PT can modulate necrosis. Thus protoporphyrin IX (PPIX), a PBR ligand, induces PT and subsequent necrosis in hepatocytes (34). Other ligands of the PBR such as PK11195 and chlorodiazepam can facilitate tumor necrosis factor-α-induced necrosis of L929 cells (35). Moreover, a number of toxic metabolites such as reactive oxygen species, nitric oxide, and supraphysiological Ca^{2+} concentrations have been shown to induce PT in several models of cell death (29, 30).

Based on this evidence, PT has been postulated to underlie mitochondrial changes occurring during the early phase of necrosis.

EVIDENCE FOR THE INVOLVEMENT OF MITOCHONDRIA IN APOPTOSIS

The absence of changes in mitochondrial ultrastructure, as well as the fact that cells lacking mitochondrial DNA (i.e. cells possessing respiration-deficient mitochondria) can undergo apoptosis (36), initially supported the idea that mitochondria do not intervene in the process of apoptosis. This interpretation is incorrect, as discussed below.

Chronological Evidence Implying Mitochondria in Apoptosis

We and others have observed that cells undergoing apoptosis exhibit a reduction of the cellular uptake of $\Delta\Psi_m$-sensitive fluorochromes [JC-1, $DiOC_6(3)$, rhodamine 123, CMXRos] (23, 24). Dissipation of the $\Delta\Psi_m$ is a general feature of apoptosis, irrespective of the cell type (neurons, fibroblasts, thymocytes, monocytes, hepatocytes, lymphocytes, tumor cells, etc) and of the apoptosis

inducer: toxins, suboptimal culture conditions, interventions on second messenger systems, ligation of cell surface receptors (Fas/Apo-1/CD95, TNF-R, etc), glucocorticoid receptor occupancy, or absence of obligatory growth factors (e.g. serum withdrawal) (2, 16, 23, 24, 37–51). The $\Delta\Psi_m$ disruption is also observed in cells lacking mitochondrial DNA (42). Whenever a pharmacological or genetic manipulation succeeds in preventing apoptosis, it also abolishes the $\Delta\Psi_m$ disruption that usually precedes cytolysis (Table 4). The $\Delta\Psi_m$ collapse constitutes an early event vis-à-vis the other manifestations of apoptosis detectable at the levels of the nucleus (chromatin condensation and DNA fragmentation, PARP cleavage), of the cytoplasma (activation of CPP32, shrinkage, calcium influx, and potassium efflux), or of the plasma membrane (phosphatidylserine exposure and later increase in permeability) (Figure 2). Nonetheless, the $\Delta\Psi_m$ collapse marks an already irreversible stage of the apoptotic process. Thus purified cells with a low $\Delta\Psi_m$ proceed to full-blown apoptosis, even after withdrawal of the apoptosis-inducing stimulus (38). Based on these findings, we conclude that the disruption of the $\Delta\Psi_m$ defines an early, irreversible stage of apoptosis. In addition to changes in the $\Delta\Psi_m$, further alterations affecting mitochondrial membrane permeability have become apparent. Thus cytochrome *c*, which is normally confined in the mitochondrial intermembrane space, is found in the cytosol of cells undergoing apoptosis. Again, this process precedes nuclear apoptosis (52–54). At present, it is a matter of debate whether cytochrome *c* release is a cause and/or a consequence of PT pore opening (53–55).

Functional Evidence Implying Mitochondria in Apoptosis

The inhibition of the first stage of pre-apoptosis (collapse of the $\Delta\Psi_m$) by cyclosporin A and *N*-methyl-Val-cyclosporin A (39, 43) established the link with PT, a mitochondrial process that is specifically inhibited by cyclophilin D ligands. We observed that the disruption of the mitochondrial transmembrane potential induced by protonophores or protoporphyrin IX entrains apoptosis (44, 46, 56). In contrast, two agents that act on mitochondria to inhibit PT, bongkrekic acid (a ligand of the adenine nucleotide translocator, another putative constituent of the megachannel) and CMXRos (which acts on mitochondrial matrix thiols), inhibit the disruption of the $\Delta\Psi_m$ as well as the later fragmentation of nuclear DNA (Table 3) (43, 44, 46, 49, 56). The inhibition of PT prevents cytolysis and all apoptotic alterations at the level of the plasma membrane, the nucleus, and the cytoplasma (43, 44, 46, 49–51, 56). These results indicate that the mitochondrial PT constitutes a critical coordinating element of the apoptotic process. This is also suggested by the fact that the oncoprotein Bcl-2 can function as an endogenous inhibitor of PT (see below).

Table 4 Glucocorticoid-induced thymocyte death: mitochondrial and nuclear parameters

Glucocorticoid receptor ligand	Inhibitor (active principle)	$\Delta\Psi_m$ disruption	Nuclear apoptosis	Cytolysis
RU38486 1-10 μM (antagonist)	None	−	−	−
Corticosterone 1 μM (natural agonist)	None	+	+	+
Dexamethasone 1 μM (synthetic agonist)	None	+	+	+
	RU38486 10 μM (GC receptor antagonist)	−	−	−
	Actinomycin D 50 μM (inhibitor of transcription)	−	−	−
	Cycloheximide 35 μM (inhibitor of translation)	−	−	−
	TLCK 100 μM (serine protease inhibitor)	−	−	−
	MG123 50 μM (proteasome inhibitor)	−	−	−
	N-acetylcysteine 30 mM (anti-oxidant)	−	−	−
	Catalase 1000 U/ml (anti-oxidant)	−	−	−
	Monochlorobiman 10 μM (thiol reactive)	−	−	−
	CMXRos 1 μM (thiol-reactive, matrix-targeted)	−	−	−
	Bongkrekic acid 50 μM (PT inhibitor)	−	−	−
	bcl-2 transgene (PT inhibitor)	−	−	−
	Z-VAD.fmk 50 μM (caspase inhibitor)	+	−	+
Partial receptor agonists with reduced trans-activation potential:				
RU 247821 1-10 μM	None	+	+	+
RU 24858 1-10 μM	None	+	+	+
RU 40066 1-10 μM	None	+	+	+

CMXRos, chloromethyl-X-rosamine; TLCK, *N*-tosyl-L-lysil chloromethylketone; Z-VAD.fmk, *N*-benzyl-oxycarbonyl-Val-Ala-Asp-fluoromethylketone. Results are from References 38, 39, 43, 49, 50, 56, or G Kroemer, unpublished data. Synthetic glucocorticoids with reduced transactivation potential are described in Reference 94.

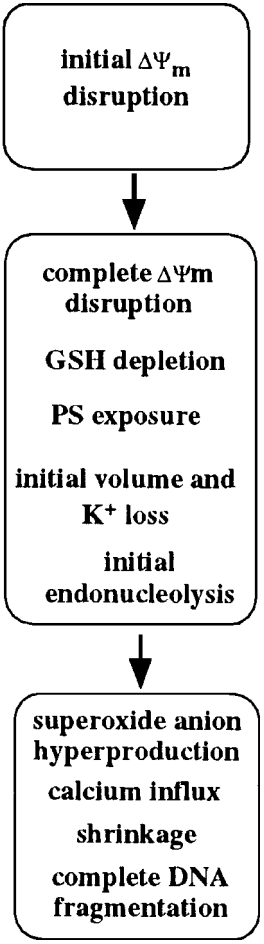

Figure 2 Chronology of some events in thymocyte apoptosis induced by glucorticoids. The first manifestation of apoptosis is a partial reduction of the mitochondrial transmembrane potential ($\Delta\Psi_m$). At the stage at which the $\Delta\Psi_m$ is completely disrupted, glutathione levels are reduced. At a later stage, cells expose phosphatidylserine residues on the surface and start to become Tunel+. It is only later that cells overproduce superoxide anion, manifest a massive Ca^{2+} influx, shrink, and demonstrate DNA loss. A similar sequence of events has been observed in response to genotoxic stimuli (e.g. etoposide, irradiation), as well as in other cell types.

Evidence from Cell-Free Systems

Taken together, the above results demonstrate that mitochondria are implicated in apoptosis. However, they do not elucidate the cause-effect relationship between mitochondrial perturbations and nuclear alterations. To show such a relationship, we have developed an experimental cell-free system in which isolated nuclei are cultured in the presence of isolated mitochondria. In analogy to a system involving *Xenopus laevis* mitochondria and nuclei (57), we found that the presence of mitochondrial products was necessary for the induction of apoptosis in nuclei from mammalian cells (42, 45, 51, 56). We have shown that mouse and human mitochondria are usually inert in such a system. Only when their megachannels are opened, can mitochondria provoke correlates of apoptotic changes (chromatin condensation, DNA fragmentation) in purified nuclei. This apoptogenic activity is due to soluble proteins liberated from the mitochondrion (42, 45, 51, 56). We have purified and analyzed one such apoptosis-inducing factor (AIF) and performed its biochemical analysis (45). AIF has no direct DNAse activity; it acts by the rapid (<5 min) activation of nuclear endonucleases. Moreover, AIF can stimulate the proteolytic activation of caspase 3 (CPP32/Yama/Apopain) (45, 51). In addition to AIF, the intermembrane protein cytochrome *c* is liberated from mitochondria undergoing PT (55). Cytochrome *c* by itself is not apoptogenic. However, it can cooperate with other factors to activate caspases and nuclear endonucleases (52).

In conclusion, it appears that apoptogenic proteases and/or protease activators are released from mitochondria upon PT and that these products are necessary for nuclear apoptosis to occur.

THE BCL-2 COMPLEX: A MITOCHONDRIAL REGULATOR

The family of Bcl-2-related proteins constitutes one of the biologically most important classes of apoptosis-regulatory gene products. Bcl-2 belongs to a growing family of apoptosis-regulatory gene products that may be either death antagonists (e.g. Bcl-2, Bcl-X_L, Bcl-w) or death agonists (e.g. Bax, Bak, Bcl-X_S, Bad) (58). The relative amount of death agonists and antagonists from the Bcl-2 family constitutes a regulatory switch whose function is determined, at least in part, by selective protein-protein interactions (59). Homologous recombination reveals that Bcl-2-like apoptosis-inhibitory proteins exert essential cytoprotective functions; their deficiency entails the ablation of determined cell types, for instance that of lymphoid cells in Bcl-$2^{-/-}$ and that of postmitotic neurons in Bcl-$X^{-/-}$ mice (58). The functional importance of these proteins is also underlined by the fact that many tumors hyperexpress apoptosis-inhibitory

Table 5 Apoptosis-inducing regimes in which Bcl-2 preserves mitochondrial function

Cell type	Inducers of apoptosis	Effect of Bcl-2 on mitochondria	Reference
Thymocytes or T cells	Glucocorticoids, ceramide,	Stabilizes $\Delta\Psi_m$	(39)
	ter-butylhydroperoxide,		(45)
	protoporphyrine IX,		(46)
	mClCCP (protonophore),		(45)
	etoposide (VP16),		(48)
	γ-irradiation, doxorubicin,		(48)
	cytosine arabinoside		(48)
T cytoplasts	Ceramide	Stabilizes $\Delta\Psi_m$	(43)
B cells	Surface IgM cross-linking,	Stabilizes $\Delta\Psi_m$	(48)
	cyclosporin A, etoposide (VP16),		(48)
	γ-irradiation, doxorubicin,		(48)
	cytosine arabinoside,		(48)
	ter-butylhydroperoxide,		Unpubl.
	ceramide, serum withdrawal		Unpubl.
PC12 cells	Cyanide, rotenone,	Stabilizes $\Delta\Psi_m$	(22)
	antimycin A, etoposide (VP16),		(22)
	calcium ionophore		(22)
Fibroblasts	p53	Stabilizes $\Delta\Psi_m$	(95)
HL60 cells	Etoposide, staurosporine	Prevents cytochrome *c* release and $\Delta\Psi_m\downarrow$	(53)

or hypoexpress death-inducing members of the Bcl-2 family. Transgene- or transfection-induced overexpression of Bcl-2 tends to protect cells against most apoptosis-inducing protocols, as well as against some necrosis-provoking regimens (58). Transfection with *bcl-2* or *bcl-*X_L inhibits or retards the early mitochondrial changes associated with apoptosis (2, 22, 39, 43, 48, 51, 60) (Table 5). This finding has been obtained in cells and in cytoplasts (anucleate cells) (43, 48). In contrast, hyperexpression of the Bcl-2 antagonist Bax causes a $\Delta\Psi$ dissipation (14). These findings suggest that Bcl-2 related proteins regulate mitochondrial functions.

Many proteins encoded by the *bcl-2* gene family are predominantly localized in the outer mitochondrial membrane, within the contact site with the inner membrane. In lymphoid cells, Bcl-2 expression correlates stoichiometrically with that of the PBR, suggesting that Bcl-2 might interact with PBR or with a PBR-associated protein (61), perhaps carnitine palmitoyltransferase I, with which Bcl-2 interacts directly (62). Several prominent members of the Bcl-2 family possess C-terminal transmembrane (TM) domains, allowing for their incorporation into intracellular membranes. Deletion mutants or replacement

of the TM domain by targeting sequences from other proteins have shown that, at least in some experimental systems, Bcl-2, Bcl-X_L, and Bax act on mitochondrial membranes, rather than on membranes of other intracellular compartments, to prevent (Bcl-2, Bcl-X_L) or induce (Bax) cell death (63, 64). In cell-free systems of apoptosis, Bcl-2 must also be present in mitochondria rather than in the nuclear envelope to inhibit apoptosis (45, 53, 54, 57). Mitochondria purified from cells hyperexpressing Bcl-2 are protected against the PT-inducing effect of several apoptosis inducers such as pro-oxidants, low doses of Ca^{2+}, protoporphyrin IX, and the cytosol of ceramide-treated cells. Concomitantly, Bcl-2 protects against the release of AIF and cytochrome *c* (45, 51, 53, 54, 56). However, Bcl-2 does not counteract the PT-inducing effect of other agents such as high concentrations of Ca^{2+} (500 μM), the thiol-cross-linking agent diamide, and high doses of recombinant caspase 1 (ICE) (45, 51, 56). Thus Bcl-2 does exert direct PT-inhibitory effects on mitochondria, although with a limited inhibitory spectrum. In contrast, Bcl-2 does not affect the formation of AIF and has only marginal, if any, effects on the action of cytochrome *c* or AIF on isolated nuclei in vitro (45, 56, 57, 65).

The three-dimensional structure of the Bcl-X_L monomer in the absence of its C-terminal hydrophobic domain (Bcl-$X_L\Delta$TM) consists of two central hydrophobic helices surrounded by five amphipathic helices, as well as a 60 residue flexible loop (66). An elongated hydrophobic pocket is created by the close spatial proximity of domains BH1, BH2, and BH3. This structure bears strong similarity to the pore-forming domains of several bacterial toxins, in particular colicins A and E1, and diphtheria toxin (66). Like these bacterial toxins, Bcl-$X_L\Delta$TM can insert into synthetic lipid vesicles or planar lipid bilayers and form an ion-conduction channel. This channel is pH sensitive (low pH opens) and becomes cation selective at physiological pH ($K^+ = Na^+Ca^{2+} > Cl^-$), at which it displays a multiple conductance state with identical ion selectivity (67). The channel-forming domains (α helices 5 and 6 between BH1 and BH2) vary between different members of the Bcl-2 family. Accordingly, Bax possesses a different ion selectivity ($Cl^- > K^+$). At present it is not clear whether Bcl-2 and Bax regulate PT directly or indirectly, for instance, by regulating other mitochondrial functions such as cytochrome *c* release (53, 54) or the activity of mitochondrial enzymes.

HOW MITOCHONDRIA SENSE DAMAGE AND INTEGRATE SPECIFIC DEATH SIGNALS

The exact composition of the PT pore complex is not known; it is currently believed to involve cytosolic proteins (hexokinase); outer membrane proteins: PBR; porin, also called voltage-dependent anion channel (VDAC);

Table 6 Functions of the permeability transition pore

Function	Principles of modulation	Example
Voltage sensor	Low $\Delta\Psi_m$ induces PT	Anoxia, respiratory inhibitors induce PT
	High $\Delta\Psi_m$ inhibits PT	Hyperpolarization (nigericin) inhibits PT
Thiol sensor	Oxidation of a critical matrix dithiol (in equilibrium with GSH) induces PT	Pro-oxidants and thiol cross-linking induce PT Prevention of thiol cross-linking prevents PT
Sensor of pyridine oxidation	Oxidation of $NAD(P)H_2$ favors PT (in equilibrium with GSH oxidation)	$NAD(P)H_2$ prevents PT Anti-oxidants prevent PT
Matrix pH sensor	Reversible histidine protonation prevents PT	Akalinization (pH = 7.3) favors PT Neutral or acidic pH inhibits PT
Cation sensor	Ca^{2+} induces PT Other divalent cations inhibit PT	Increase in matrix Ca^{2+} induces PT Mg^{2+} and Zn^{2+} prevent PT
ADP/ATP sensor	ADP (and ATP) inhibit PT	Extra ATP (glycolytic substrates) prevents PT Oligomycin (F_1 ATPase inhibitor) induces PT
Protease sensor?	Direct action of proteases on outer membrane proteins	Caspase 1 induces PT Calpain-like enzyme may induce PT
Lipid acid sensor?	Long chain lipid acids induce PT	Palmitate and stearate induce PT Carnitine prevents PT
Peptide sensor?	Amphipathic peptides induce PT	Mastoparan induces PT

intermembrane proteins (creatine kinase); at least one inner membrane protein (the adenine nucleotide translocator (ANT); and at least one matrix protein (cyclophilin D) (29–33). It may also interact with proteins of additional multiprotein complexes, namely the Tim (transporter of the inner membrane) complex, the Tom (transporter of the outer membrane) complex (68), and the Bcl-2 complex (58).

Irrespective of its exact composition, the PT pore complex contains multiple targets for pharmacological interventions and is regulated by numerous endogenous physiological effectors (Table 6, Figure 3). Such effectors include ions (divalent cations, mainly Ca^{2+} and Mg^{2+}); protons; the $\Delta\Psi_m$; the concentration of adenine nucleotides (ADP, ATP) (29, 30); the pyrimidine redox state (NAD versus $NADH_2$; NADP versus $NADPH_2$); the thiol redox state (controlled by glutathione) (69); reactive oxygen species and nitric oxide (70, 71);

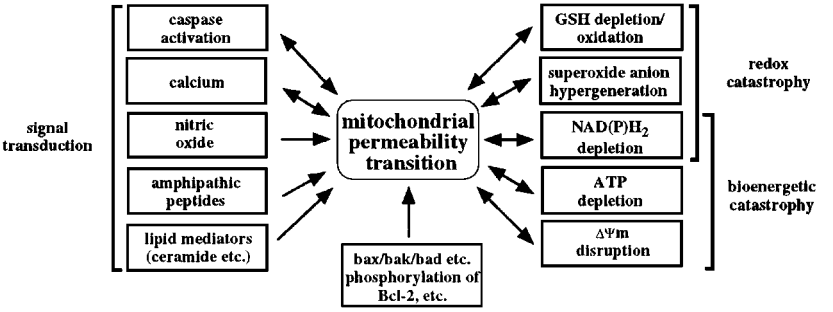

Figure 3 Inducers of permeability transition. Different signal transduction pathways can promote the activation of caspases (e.g. cross-linking of Fas/Apo-1/CD95), increases in cytosolic Ca^{2+} levels (e.g. excitotoxins such as glutamate), nitric oxide, amphipathic peptides (e.g. after withdrawal of NGF), or lipid mediators (e.g. ceramide and sphingosine) can provoke PT. In addition, changes in the composition of the Bcl-2 complex such as hyperexpression of the Bcl-2 antagonist Bax (e.g. in response to p53 overexpression after genotoxic insults) or phosphorylation-mediated inactivation of Bcl-2 (e.g. after treatment with the microtubule disrupting agent taxol) can induce PT. Major changes in the cellular redox balance or in bioenergetic parameters can also trigger PT. Note that, in several cases, PT is a self-amplifying process (*two-way arrows*).

the concentrations of lipoids (lipid acids, acyl-CoA, ceramide and derivatives) (29, 30); the concentrations of determined peptides (amphipathic peptides and perhaps signal sequences of peptides targeting proteins to the mitochondrial import machinery) (72, 73); and changes in the composition or function of the Bcl-2 complex (39, 56, 58). As a rule, it appears that any major change in energy balance (absence of oxygen, depletion of ATP and ADP, depletion of $NAD(P)H^2$, disruption of the $\Delta\Psi_m$) or changes in the redox balance (oxidation/depletion of non-oxidized glutathione or $NAD(P)H_2$, hyperproduction of reactive oxygen species) can provoke PT. This implies that PT integrates stress responses and that major damage of cells will invariably cause PT. Moreover, because PT itself causes such changes in energy and redox balance (see below), massive PT locks the cell into an irreversible stage.

In addition, determined signal transduction pathways triggered via intracellular or cell surface receptors can result in PT. Thus PT is facilitated by a number of second messengers: increases in cytosolic Ca^{2+} concentration, ceramide, and caspase 1-like enzymes such as ICE (interleukin 1β converting enzyme). Because PT is regulated by the Bcl-2 complex, changes in the molecular stoichiometry of this complex, e.g. enhanced synthesis of the Bcl-2 antagonist Bax, or signal transduction pathways, culminating in post-translational modifications of the Bcl-2 complex, can also facilitate PT. The phosphorylation of Bcl-2 or Bcl-2 homologues, e.g. Bad, as well as the subcellular distribution

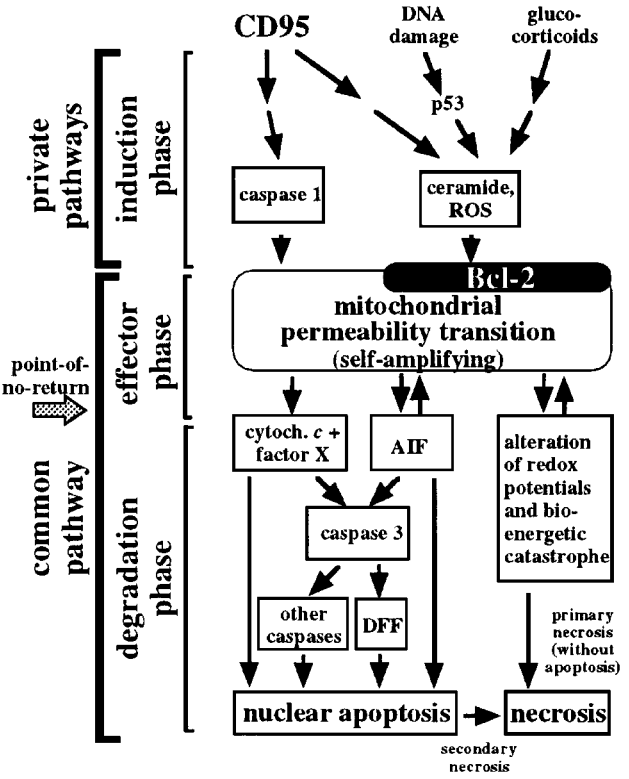

Figure 4 Consequences of permeability transition. Different death-triggering pathways employ distinct (private) signal transduction pathways that will culminate in the induction of permeability transition (PT). Thus PT induced by DNA damage requires p53 activation, whereas induction of PT by ligation of Fas/Apo-1/CD95 requires the activation of ICE-like caspases. PT constitutes the first rate-limiting event of the common pathway of apoptosis and can be induced either in a Bcl-2-regulated or in a Bcl-2-independent fashion. Thus ceramide-induced PT is inhibited by overexpression of Bcl-2. In contrast, PT induced by caspase 1-like proteases is not inhibited by Bcl-2. Upon PT, apoptogenic factors are released from the mitochondrial intermembrane space and leak into the cytosol. At least two such factors have been characterized: cytochrome *c* (which requires unknown cytosolic factors to activate caspase-3 and to induce nuclear apoptosis) and AIF, which suffices to induce nuclear apoptosis in vitro. Different proteases from the caspase family can participate in the apoptotic degradation phase downstream of PT. In addition, PT causes major changes in cellular redox potentials (depletion of non-oxidized glutathione, depletion of $NAD(P)H_2$, hyperproduction of superoxide anion), energy metabolism (depletion of $NAD(P)H_2$ and ATP), and ion compartmentalization (outflow of Ca^{2+} from the matrix and later Ca^{2+} efflux from the endoplasmic reticulum and influx via the plasma membrane). The scheme provides an explanation for the choice between apoptosis and necrosis. Massive induction of PT with subsequent rapid depletion of energy-rich phosphates causes primary necrosis, i.e. the disruption of plasma membrane integrity before apoptogenic proteases come into action. In contrast, a more subtle, regulated induction of PT allows for the activation and action of proteases, thus giving rise to the apoptotic phenotype.

of Bcl-2-related proteins, e.g. Bad, or Bcl-2-associated proteins, e.g. Bag-1, Raf-1, is regulated by growth factor receptors (58, 74, 75), thus suggesting how growth factor withdrawal can trigger PT indirectly, via changes in the composition of the PT-regulatory Bcl-2 complex (58).

Together these findings indicate, that in addition to integrating various damage responses, PT can be triggered via receptor-connected pathways. PT may constitute the crossroad of both nonspecific damage responses and responses mediated via specific receptors (Figure 4).

HOW MITOCHONDRIA AND THEIR PRODUCTS EXECUTE CELL DEATH

Under normal circumstances, the inner mitochondrial membrane is nearly impermeant. This feature is required for maintaining the inner transmembrane potential ($\Delta\Psi_m$). Opening of the PT pore allows the diffusion of solutes with a M_W of >1500 kDa, according to gross estimations based on the use of polyethylene glycol polymers (29, 30). The result evinces an immediate dissipation of the $\Delta\Psi_m$, with consequent loss of mitochondrial RNA and protein synthesis, cessation of the import of most proteins synthesized in the cytosol (which depends on the $\Delta\Psi_m$), 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 hyperproduction of superoxide anion on the uncoupled repiratory chain. Accordingly, multiparameter fluorescence analyses reveal that the $\Delta\Psi_m$ collapse is closely linked to major changes in cellular redox potentials, namely $NAD(P)H_2$ depletion (40), GSH depletion/oxidation (50), and later increases in superoxide anion generation (39) and massive cytosolic Ca^{2+} elevations (50). Intriguingly, these consequences of PT themselves can provoke PT (29, 30), suggesting the existence of one or several self-amplifying feedback loops that may explain why PT (and cell death) is occurring as an all-or-nothing phenomenon.

The bioenergetic and redox changes of PTs themselves are sufficient to cause necrosis. But how does PT trigger apoptosis? PT allows for the release of proteins usually confined to the mitochondrial compartment. Thus PT causes the release of cytochrome *c* from the intermembrane space into the cytosol (55). The protein precursor of cytochrome *c* (apocytochrome *c*) is synthesized in the cytosol and transported into the intermembrane space, where the heme lyase attaches a heme group to generate holocytochrome *c*. Neither cytochrome *c* nor its precursor apocytochrome *c* itself is apoptogenic, but holocytochrome *c*, which lacks a heme group, can interact with other yet unknown cytosolic factors to activate the caspases 3 (CPP32/Yama/Apopain) and 6 (Mch-2) and to induce nuclear apoptosis in vitro (52). CPP32 can then activate DNA fragmentation factor

(DFF), which in turn acts to activate nucleases (76). In addition to cytochrome *c*, mitochondria undergoing PT release AIF, an unstable protein of approximately 50 kDa, that suffices to cause nuclear apoptosis and activation of CPP32 in cell-free systems (45, 51). As is true for cytochrome *c*, this activity is pre-formed. Exhaustive studies have identified an inhibitor of AIF: *N*-benzyloxycarbonyl-Val-Ala-Asp.fluoromethylketone (Z-VAD.fmk) (45). This protease inhibitor abolishes all activities of AIF on the nucleus. Z-VAD.fmk is also a universal inhibitor of nuclear apoptosis, occurring in intact cells, irrespective of the cell type (3, 77), thus emphasizing the possible in vivo relevance of AIF. In summary, mitochondria contain several proteins endowed with the capacity of stimulating at least some facets of the apoptotic program in cell-free systems. At present, it appears that at least two biochemical pathways may link mitochondria to nuclear apoptosis: AIF and cyctochrome *c* plus factor X $\rightarrow$ caspase 3 $\rightarrow$ DFF.

If PT can stimulate both primary necrosis and apoptosis, what does make the difference? As a possibility, the bioenergetic and redox catastrophe ensuing PT (which would induce necrosis) and the activation of catabolic enzymes (caspases and nucleases) might compete with each other in a sort of race. Cells would only die from primary necrosis when apoptogenic proteases fail to come into action, either because they are inhibited (e.g. by addition of Z-VAD.fmk) or because the time frame of the process is too rapid to allow for protease activation. This view of cell death would be compatible with the fact that many substances induce apoptosis at low doses (when PT is induced smoothly and cells can activate proteases) but induce necrosis at higher doses (when PT is caused abruptly and cells lyse before proteases come into action). Finally, it would explain how maintenance of high ATP levels can favor apoptosis over necrosis (12, 13), whereas inhibition of proteases or manipulations that reduce cellular ATP levels favor necrosis over apoptosis (12–16).

CONCLUSIONS AND PERSPECTIVES

The data discussed in this review indicate that mitochondria play a major role in the regulation of both physiological and pathological cell death. The available data are compatible with our current working hypothesis that mitochondrial PT (or an event closely linked to PT) is a central coordinating event of apoptosis and necrosis. This hypothesis predicts that various damage pathways and pro-apoptotic signal transduction cascades converge at the level of PT, in line with the fact that PT can be induced by numerous physiological effectors. Once PT has been triggered, a series of common pathways of cell death are initiated, each of which may be lethal. Thus PT has at least two major consequences: (*a*) a bioenergetic and redox catastrophe disrupting cellular metabolism and

(*b*) liberation of protease and endonuclease activators from mitochondria. Depending on which of these processes wins the race, either primary necrosis (lysis before activation of catabolic enzymes) or apoptosis (activation of proteases and endonucleases before lysis) ensues.

The central role of PT in the death process might allow for an operative reinterpretation of a number of hitherto controversial and apparently contradictory observations. Cell death control must function in an on/off fashion rather than in a gradual one. To explain the all-or-nothing nature of apoptosis switching, it is tempting to conceive one or several positive feedback loops in which the consequences of the apoptotic process themselves stimulate the action of the central executioner. Thus once beyond a threshold value, self-amplification would drive the cell into an irreversible death program. Several of the metabolic consequences of mitochondrial PT are well-known by-products of apoptosis: uncoupling of the respiratory chain with hyperproduction of reactive oxygen species (ROS) (39), disruption of mitochondrial (and later extra-mitochondrial) calcium homeostasis (29), and liberation of protease activators with loss of cytochrome *c* (which blocks the repiratory chain) (45, 52, 55). All these consequences of PT themselves favor PT (Figures 3 and 4). This suggests a dual role for certain molecules (Ca^{2+}, ROS, caspases) in apoptosis: as facultative constituents of signal transduction pathways initiating the vicious cycle of PT and as constant by-products of the death process.

Future studies will have to explore numerous incognita: the exact nature of the PT-inducing trigger in different pathways of apoptosis induction; the molecular composition of the PT pore complex; the interactions between the PT pore and the Bcl-2 complex; and the mechanism of how the PT pore is fine-tuned in a cell type-, differentiation-, and activation-dependent fashion. Further investigation of PT and its regulation should furnish invaluable information on the normal physiology of cell death, and by consequence pave the way for the rational design of novel cytoprotective and cytotoxic drugs. Moreover, it will be most important to understand the relationship between apoptosis, PT, and mitochondrial damage as it accumulates during aging (78) or chronic degenerative diseases (79).

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