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ABSTRACT

Programmed cell death (PCD) serves as a major mechanism for the precise regulation of cell numbers, and as a defense mechanism to remove unwanted and potentially dangerous cells. Despite the striking heterogeneity of cell death induction pathways, the execution of the death program is often associated with characteristic morphological and biochemical changes termed apoptosis.

The central components of the intrinsic apoptotic pathway involve specific proteases (i.e. caspases) and mitochondria (Nunez et al., 1998; Raff, 1998). Although for a long time the absence of mitochondrial changes was considered as a hallmark of apoptosis, mitochondria appear today as the central executioner of programmed cell death. This crucial position of mitochondria in programmed cell death control is not due to a simple loss of function (deficit in energy supplying), but rather to an active process in the regulation of effector mechanisms. This role is reinforced by the observation that mitochondria contribute to PCD signaling via the production of reactive oxygen species, as shown in TNF-α or ceramide-induced cell death during which increased mitochondrial ROS production appears as an early event of the induction phase. Recent other reviews cover the abundant literature dealing with the role of mitochondrion in cell death (Bernardi et al., 1998; Bernardi et al., 1999; Mignotte and Kroemer, 1999; Mignotte and Vayssière, 1998; Mignotte and Vayssière, 1999; Reed et al., 1998; Susin et al., 1998). In this chapter, we examine on the one hand the data concerning the mitochondrial proteins involved in PCD, and on the other hand the role of reactive oxygen species (ROS) in mitochondrial features of apoptosis.

1 Introduction

The maintenance of pluricellular organisms implies that cell death can be an important part of life. First observed during amphibian metamorphosis, normal cell death was soon found to occur in many developing tissues in both invertebrates and vertebrates (Clarke and Clarke, 1996). The term programmed cell death (PCD) was used to describe the cell deaths that occur in predictable places and at predictable times during development, to emphasize that the deaths are somewhat programmed into the development plan of the organism.

Apoptosis is a process whereby cells activate an intrinsic cell suicide program that is one of the potential cellular responses, such as differentiation and proliferation. It has been defined in 1972 by Kerr et al. in contrast to necrosis, which is a cell death generally due to aggressions from the external medium (Kerr et al., 1972).

The apoptotic process is associated with characteristic morphological and biochemical changes, such as membrane blebbing, cell shrinkage, chromatin condensation, DNA cleavage and fragmentation of the cell into membrane-bound apoptotic bodies whose surface expresses potent triggers for phagocytosis. However, it must be kept in mind that although apoptosis is the most common form of PCD, dying cells may follow other morphological types (Clarke, 1990; Schwartz et al., 1993). Moreover, apoptotic cells do not always harbor all cardinal features of their cell death type, in particular DNA cleavage does not appear to be an absolute requirement (Schulze-Osthoff et al., 1994b). Nevertheless, the observation that most cells undergoing PCD change similarly has suggested that apoptosis reflects the operation of an intracellular death program that can be activated or inhibited by a variety of physiological or pathological environmental stimuli.

Apoptosis is then a major mechanism for the precise regulation of cell numbers, and unwanted and potentially dangerous cells removal (Raff, 1992; Vaux et al., 1994; Williams, 1991). In addition to the beneficial effects of apoptosis in development and tissue homeostasis, the inappropriate activation of cell death may cause or contribute to a variety of diseases (Thompson, 1995), including acquired immunodeficiency syndrome (AIDS) (Ameisen et al., 1995),

neurodegenerative disorders, and ischemic strokes (Martinou, 1993; Raff et al., 1993). Conversely, a defect in apoptosis activation could be responsible for some autoimmune diseases (Tan, 1994) and is also involved in oncogenesis (Bursch et al., 1992).

Cascade of events leading to apoptosis can be divided into several phases (see (Mignotte and Kroemer, 1999; Mignotte and Vayssière, 1999)). During the activation phase, multiple signaling pathways lead from the various death-triggering signals (lineage information, extracellular survival factors or signals, cell interactions, hormones, cell contacts, genotoxic and physical trauma, anoxia, oncogene expression, immune killing…), to the central control of the cell death machinery and activate it. This is followed by the execution stage, in which the activated machinery acts on multiple cellular targets, and, finally, the destruction phase in which the dead or dying cell is broken down.

2 Intracellular control of the cell death program

The existence of an intrinsic cell suicide program was ascertained through genetic studies in the nematode *Caenorhabditis elegans* that identified genes involved in the cell death program and its control (Ellis and Horvitz, 1986; Horvitz and Ellis, 1982), and then through the finding that some of these genes were homologous to mammalian genes (Hengartner and Horvitz, 1994; Yuan et al., 1993). These considerations have led to a different meaning of the term apoptosis: it now refers to any cell death that results from an intracellular death program, no matter what activates it and whatever the changes associated with the destruction of the cell. In this way PCD appears as a critical element in the repertoire of potential cellular responses, as are cell differentiation, quiescence or proliferation. Moreover, these studies demonstrated the very early occurrence of PCD in the course of metazoan evolution and the substantial conservation of its basal machinery from nematodes to humans.

Genetic studies in *C. elegans* were a major contribution in the understanding of the molecular nature of this death program, leading to the identification of a dozen of cell death genes (ced) (Ellis et al., 1991). *Ced-3* and *ced-4* are essential for cell death, whereas *ced-9* antagonises the activities of *ced-3* and *ced-4*, and thereby protects cells that should survive from any accidental activation of the death program. Caspases (cysteinyl aspartases) are the mammalian homologues of CED-3 and form a family of proteases implicated in apoptosis (Yuan, et al., 1993) (see table 1). The mammalian caspase family now comprises at least fourteen known members, most of which have been definitively implicated in PCD (for review see(Cryns and Yuan, 1998)). All cleave their substrates after specific aspartic acids and are themselves activated by cleavage at specific aspartic acids (Nicholson and Thornberry, 1997). Caspases mediate apoptosis by cleaving selected intracellular proteins, including proteins of the nucleus, nuclear lamina, cytoskeleton, endoplasmic reticulum, and cytosol.

3 The Bcl-2 family

CED-9 protein is homologous to a family of many members termed the Bcl-2 family (Bcl-2s) in reference to the first discovered mammalian cell death regulator (for review see (Adams and Cory, 1998)). The Bcl-2 family proteins are involved in positive and negative regulations of apoptotic cell death (review in (Gross et al., 1999a); see table 1). Among the anti-apoptotic members, Bcl-2 and Bcl-X_L are negative regulators of cell death, able to prevent cells from undergoing apoptosis induced by various stimuli in a wide variety of cell types (Korsmeyer, 1992; Zhong et al., 1993), whereas others, such as Bax, Bid and Egl-1 promote or accelerate cell death. CED-9, Bcl-2 (Akao et al., 1994; Chen et al., 1989; de Jong et al., 1994; Hockenbery et al., 1990; Janiak et al., 1994; Krajewski et al., 1993; Nakai et al., 1993; Nguyen et al., 1993), Bcl-xL

(Gonzalez-Garcia et al., 1994), Mcl-1 (Wang and Studzinski, 1997; Yang et al., 1995), the BHRF1 Epstein-Barr virus protein (Hickish et al., 1994) and probably other members of the Bcl-2 family are localized to the cytoplasmic surfaces of the nuclear envelope, the endoplasmic reticulum and the outer mitochondrial membrane. It must be yet underlined that only a fraction of Bcl-x_L resides on membranes, even though it bears a C-terminal membrane anchor involved in the targeting to membranes (Nguyen, et al., 1993). Moreover, this hydrophobic C-terminus is not involved in the association with the membrane in the case of Bax (Goping et al., 1998; Tremblais et al., 1999).

The membrane association of Bcl-2 is of functional significance as mutant Bcl-2 molecules lacking this membrane anchorage capacity are less effective at preventing apoptosis in some systems (Borner et al., 1994; Nguyen et al., 1994; Zhu et al., 1996). Indeed, it has been reported that, in inhibiting apoptosis of MDCK cells, a mutant Bcl-2 molecule whose anchorage is targeted specifically to the mitochondria is as effective as the wild type protein, whereas mutant Bcl-2 targeted to the endoplasmic reticulum loses this capacity (Zhu, et al., 1996). In contrast, Bcl-2 targeted to the endoplasmic reticulum in the Rat-1/myc fibroblasts proved to be more active than when targeted to mitochondria. Thus, Bcl-2 mutants with restricted subcellular location reveal distinct pathways for apoptosis depending on cell type. When associated to the endoplasmic reticulum membrane, Bcl-2 could be involved in maintenance of the calcium homeostasis (Distelhorst et al., 1996; He et al., 1997; Lam et al., 1994), while it could modulate protein subcellular trafficking through nuclear pores (Ryan et al., 1994).

Bcl-2s proteins can dimerize with one another, with one monomer antagonizing or enhancing the function of the other. In this way, it is assumed that the ratio of activators to inhibitors in a cell could determine the propensity of the cell to undergo apoptosis (Korsmeyer, 1995). Nevertheless, it has been suggested that all the heterodimers are not formed in the cells (Hsu and Youle, 1998). The mechanism(s) by which proteins of the Bcl-2 family modulate apoptosis is not yet well known and several conflicting theories have been proposed. In non-stimulated cells, monomeric Bax is located in the cytosol and in peripheral association with intracellular membranes including mitochondria, but inserts into mitochondrial membranes after a death signal (Gross et al., 1998; Wolter et al., 1997). A widely accepted model postulates that homodimers of Bax promote apoptosis (Finucane et al., 1999; Gross, et al., 1998; Gross, et al., 1999a; Pastorino et al., 1998; Xiang et al., 1996), and that the functional effect of Bcl-2 related proteins is to form competing heterodimers with Bax that cannot promote apoptosis (Oltvai et al., 1993; Sedlak et al., 1995). However, in some systems, Bax binding by Bcl-2 was not sufficient to prevent apoptosis and the overexpression of Bcl-2 or Bcl-x_L can repress apoptosis in the absence of Bax (Cheng et al., 1996; Knudson and Korsmeyer, 1997). Moreover, mechanisms other than the simple dimerization among members of the Bcl-2 family may be required for the regulation of apoptosis (Hsu and Youle, 1998). Thus, while an *in vivo* competition exists between Bax and Bcl-2, each is able to regulate apoptosis independently.

In both worm and mammalian cells, the anti-apoptotic members of the Bcl-2 family act upstream of the "execution caspases", somehow preventing their proteolytic processing into active killers (Golstein, 1997; Shaham and H.R., 1996). Some proteins, such as Bap31 in the endoplasmic reticulum, may bind caspases and Bcl-2s family members, coordinating thus their activities (Ng et al., 1997). Furthermore, two main mechanisms of action have been proposed to connect Bcl-2s to caspases. In the first one, Bcl-2 would act by regulating the release from mitochondria of apoptogenic factors, such as the Apoptosis-Inducing-Factor (AIF), some caspases activators (e.g. cytochrome c) and pro-caspases from mitochondria to cytosol (Kluck et al., 1997a; Susin et al., 1996; Yang et al., 1997). Indeed, it was shown that 1) some Bcl-2s proteins have pore-forming properties with distinct ion-conducting properties, raising the possibility that these products can regulate the permeability of the intracellular membranes (Minn et al., 1997; Muchmore et al., 1996; Schendel et al., 1997) ; 2) activation of "execution caspases" required the preliminary shift to the

cytosol of regulatory components, such as cytochrome c or pro-caspases, previously sequestered in mitochondria (Kluck, et al., 1997a; Krippner et al., 1996; Susin, et al., 1996; Yang, et al., 1997).

An alternative model proposes that the pro-survival proteins may function downstream the release of apoptogenic factors, by directly inhibiting the ability of CED-4 like proteins to activate caspases. This model arose from the elucidation of the role of the somewhat mysterious CED-4 protein in the nematode (Hengartner, 1997). Indeed, it was first shown that CED-9 interacts with CED-3 via the bridging protein CED-4 that binds simultaneously to CED-9 and CED-3. CED-9 prevents CED-4 from inducing proteolytic processing and activation of CED-3. An equivalent ternary complex was found to be present in mammalian cells involving the mammalian equivalent of CED-4, Apaf-1 (apoptosis protease-activating factor 1, (Zou et al., 1997), caspase-9 and Bcl-x_L, in which Bcl-x_L inhibits Apaf-1-mediated maturation of caspase-9 (Chinnaiyan et al., 1997). In this paradigm, pro-apoptotic relatives like Bik may free CED-4/Apaf-1 from the death inhibitor. Nevertheless, Moriishi et al. have recently proposed that the prosurvival Bcl-2 homologues do not act by sequestering Apaf-1, but should constrain its activity indirectly (Moriishi et al., 1999). Additionally, there is some evidence that binding of pro-survival proteins to the apoptosome complex alter its location in cells, pulling it from the cytosol to the intracellular membranes where Bcl-2s often reside (Chinnaiyan, et al., 1997; Wu et al., 1997).

These distinct and probably independent links between Bcl-2 and caspases match with the apparent redundancy of caspases and the existence of several alternative activation pathways that seem to be the rule for mammalian cell death. The multiplicity of caspases illustrates this complexity. Although caspases appear as the key components of the execution machinery of the cell death program, they also intervene in the death signal transduction cascade in some PCD, such as those triggered by surface receptor activation (Nagata, 1997). These different possible pathways of caspase activation would be consistent with the previously described possibility for some caspases to be activated by autoprocessing and for others to require transcleavage (the proteolytic cascade). Given the complexity and diversity of caspase activation pathways, these experiments point to the mitochondrion as a unifying element of apoptosis. An irony in the story of PCD studies, for a long time the absence of mitochondrial changes was considered as a hallmark of apoptosis (Kerr and Harmon, 1991) while, conversely, mitochondria appear today as the central executioner of programmed cell death (Golstein, 1997; Kroemer et al., 1997; Reed, 1997).

4 Mitochondrial events

4.1 A decrease in mitochondrial membrane potential precedes DNA fragmentation during apoptosis

Several changes in mitochondrial biogenesis and function are associated with the commitment to apoptosis. A fall of the membrane potential ($\Delta\Psi m$) occurs before the fragmentation of the DNA in oligonucleosomal fragments (Kroemer et al., 1995; Petit et al., 1995; Vayssière et al., 1994; Zamzami et al., 1995a; Zamzami et al., 1995b). This drop of $\Delta\Psi m$ is responsible for a defect of maturation of mitochondrial proteins synthesized in the cytoplasm (Mignotte et al., 1990), cessation of mitochondrial translation and an uncoupling of the oxidative phosphorylations (Vayssière, et al., 1994). The drop of $\Delta\Psi m$ is detectable whatever the cellular type, or the apoptosis induction signal and marks the point of no-return of a cell condemned to die (Kroemer, et al., 1995).

The fall of $\Delta\Psi_m$ could be due to the permeability transition (PT), a phenomenon that is characterized by the opening of large conductance channels (mitochondrial PT pores) and by its sensitivity to very low concentration of cyclosporine A (Zoratti and Szabo, 1995, Zamzami, 1998 #2854). These pores, permeable to compounds of molecular mass up to 1500 Da are formed under specific conditions, but their molecular composition remains elusive. The peripheral benzodiazepin

receptor, that has recently been implicated in the protection against ROS (Carayon et al., 1996), creatine kinase and the voltage dependent anion channel (VDAC), the translocase of adenine nucleotides (ANT) and the mitochondrial matrix cyclophilin are probable components of the PT pore (Hirsch et al., 1998; Zamzami et al., 1996a).

The opening of these PT pores in the inner membrane allows for an equilibration of ions within the matrix and intermembrane space of mitochondria, thus dissipating the H+ gradient across the inner membrane and uncoupling the respiratory chain. These events lead to the decrease of the $\Delta\Psi_m$, the arrest of ATP synthesis (Bernardi et al., 1992; Petronilli et al., 1994), and a volume dysregulation of mitochondria due to the hyperosmolarity of the matrix, which cause the matrix space to expand. Because the inner membrane a larger surface area than the outer membrane, this matrix volume expansion can eventually provokes outer membrane rupture, releasing intermembrane space molecules into the cytosol. Furthermore, permeability transition has properties of self-amplification: the drop of the $\Delta\Psi_m$ that is linked to depletion of non-oxidized glutathione (Macho et al., 1997), and the opening of the PT pores would increase the permeability transition in a retrograde manner (Ichas et al., 1997). We have therefore proposed that the opening of the PT pore may constitute an irreversible state of apoptosis and could account for the apparent synchronization in the drop of $\Delta\Psi_m$ that takes place simultaneously in all the mitochondria of a same cell (Kroemer, et al., 1995). Altogether, these results sustain the hypothesis that the opening of PT pores is involved in the disruption of $\Delta\Psi_m$ observed during apoptosis, at least during the effector phase (Kroemer, et al., 1997). For a review about PTP, see (Bernardi, et al., 1999; Lemasters et al., 1998) and references within.

Moreover, alterations of mitochondria can induce apoptosis (Hartley et al., 1994; Wolvetang et al., 1994), and direct interventions on the mitochondrial permeability modulate apoptosis. The links between mitochondrial perturbations and nuclear alterations have been studied by means of acellular systems where purified nuclei and purified mitochondria are confronted (Newmeyer et al., 1994). Such a system allows studying reciprocal and direct effects of one organelle on another and to characterize at the biochemical level the factors involved.

These experiments have shown that when mitochondria are treated with substances capable to induce PT pores opening, they provoke nuclear apoptosis (condensation of the chromatin and fragmentation of the DNA) (Zamzami et al., 1996b). A correlation between induction of the PT and nuclear apoptosis has been observed by using a variety of known inductors of the PT such as atractyloside, pro-oxidants, calcium, protonophores and substances that provoke linkage of thiol groups such as diamide. These substances, that have no direct effect on nuclei in absence of mitochondria, confer pro-apoptotic properties upon mitochondria. The pro-apoptotic character (induction of nuclear apoptosis) of the mitochondria treated with atractyloside is altered by inhibitors of the PT such as bongkrekic acid, cyclosporine A and substances like monochlorobimane that block the cross-linking of the thiols. Cyclosporine A can be replaced by its non-immunosuppressor analogue, N-methyl Val-4-cyclosporine A, which shows that its inhibitory effect on PT and nuclear apoptosis is independent from its calcineurine activity. These results suggest the implication of the PT pores opening in the regulation of apoptosis induced via the mitochondria.

Nuclei and mitochondria have been purified from hybridomas of T cells transfected by bcl-2 to study how Bcl-2 suppresses apoptosis in in vitro experiments (Zamzami, et al., 1996b). Upon treatment with atractyloside, in contrast to mitochondria purified from control cells, mitochondria purified from cells transfected by bcl-2 do not provoke nuclear apoptosis. On the contrary, nuclei purified from cells transfected by bcl-2 show a condensation of the chromatin and a fragmentation of the DNA when they are confronted to control mitochondria treated with atractyloside. Furthermore, bcl-2 inhibits the induction of permeability transition by agents such as atractyloside, oxidants and protonophores. These results show that, even if Bcl-2 intervenes also during latter apoptotic events (Guénal et al., 1997; Marton et al., 1997) and on $\Delta\Psi$m loss induced by other

mechanisms (Shimizu et al., 1998), at least a part of its activity is exerted by acting on the mitochondrial permeability transition (Decaudin et al., 1997; Susin, et al., 1996). It has also been reported that the cell death caused by overexpression of Bax could be prevented by a cyclosporin treatment (Bradham et al., 1998; Pastorino, et al., 1998), suggesting that bax could act on the PT opening (Shimizu, et al., 1998). However, these findings are still controversious because some studies indicate that the mitochondria effects of Bax are not countered by cyclosporin (Eskes et al., 1998).

Moreover, the structure of a protein of the Bcl-2 family (Bcl-x_L) has been established (Muchmore, et al., 1996). It recalls that of bacterial toxins, especially the diphtheria toxin, that forms a pH-sensitive transmembrane channel. Furthermore, the pro-apoptotic Bax protein can form channels (Antonsson et al., 1997), as reported also for the anti-apoptotic proteins Bcl-x_L (Minn, et al., 1997) and Bcl-2 (Schendel, et al., 1997). However, the intrinsic properties of Bax and those of Bcl-x_L and Bcl-2 reveal differences. The channel forming activity of Bcl-x_L and Bcl-2 is observed at highly acidic pH while Bax form channel in a wide range of pH including at pH=7, those found in cells. Furthermore, Bcl-2 can block the pore-forming activity of Bax. (Gross, et al., 1998). These results strengthen the hypothesis that these proteins are constituents of the mitochondrial PT pores. Bax might promote cell death by allowing the efflux of ions and small molecules across the mitochondrial membranes, thus triggering permeability transition, while Bcl-2 might counteract this effect.

4.2 Release of apoptogenic factors

One of the major roles of mitochondria in apoptosis is linked to the release of apoptogenic molecules from this organelle into the cytosol, such as cytochrome c and AIF (see below). Recently, the release of procaspase-2 and procaspase-9 from mitochondria was also observed during the opening of the permeability transition pores (Susin et al., 1999a). Pro-caspase-3 is found in both cytosol and mitochondria (Mancini et al., 1998), and its activation from the mitochondrial pool is regulated by Bcl-2 (Krebs et al., 1999). Translocation of active caspases from cytosol to mitochondria occurs in some situations (Chandler et al., 1998). Pro-apoptotic Bcl-2s members translocate within the cells and undergo post-traductional modifications , such as phosphorylation/dephosphorylation or cleavage, under apoptotic stimulus (Wang et al., 1999)

4.2.1 Cytochrome c release

Although apoptosis can occur in the absence of detectable cytochrome c release (Chauhan et al., 1997; Gross et al., 1999b; Tang et al., 1998), efflux of cytochrome c from mitochondria appears to be a critical coordinating step in the killing program towards which converge the multiple signaling pathways and beyond which are initiated the entire panel of apoptotic features, pro-caspase 3 expressing cells being then irreversibly committed to die (Li et al., 1997a). Translocation of cytochrome c from mitochondria to cytosol has been shown to be a crucial step in the activation of the PCD machinery in various death models, including Fas-, UV-, staurosporine or etoposide-treated mammalian cells, in a cell-free system using *Xenopus* egg extracts or dATP-primed cytosols of growing cells (Kluck, et al., 1997a; Krippner, et al., 1996; Liu et al., 1996; Yang, et al., 1997). Furthermore, direct microinjection of cytochrome c into the cytosol can induce apoptosis in various cell types (Li, et al., 1997a; Zhivotovsky et al., 1998). Cytochrome c is an essential component of the mitochondrial respiratory chain: it accepts an electron from cytochrome c reductase and passes it on to cytochrome c oxidase. It is a soluble protein that is located in the intermembrane space and is loosely attached to the surface of the inner mitochondrial membrane. Cytochrome c is translated on cytoplasmic ribosomes as apocytochrome c and follows a unique pathway into mitochondria that does not require the signal sequence, electrochemical potential and general protein translocation machinery (Mayer et al., 1995). The apoprotein, on entry into the intermembrane space, gains an

heme group, to become the fully folded holocytochrome c. This globular, positively charged protein can no longer pass through the outer mitochondrial membrane and is thought to become electrostatically attached to the inner membrane.

Released of cytochrome c into the cytosol induces nuclear apoptosis. Cytosolic cytochrome c forms an essential part of the vertebrate "apoptosome" which is composed of cytochrome c, Apaf-1 and procaspase-9 (Li et al., 1997b; Zou, et al., 1997). The result is the activation of Apaf-1, which activates proteolytic processing and activation of caspases to orchestrate execution of cell death.

Although efflux of cytochrome c from mitochondria appears to be a crucial step in the killing cascade initiated by a wide range of apoptogenic stimuli, it must be underlined that this event is not an universal requirement for death signal transduction. Indeed, on the one hand, apoptosis can occur in the absence of detectable cytochrome c release (Chauhan, et al., 1997; Tang, et al., 1998) and, on the other hand, under certain circumstances, cytochrome c release is not sufficient to promote cell death (Li, et al., 1997a). In contrast, recent studies indicate that cytochrome c is not released in some cell lines committed to cell death, but its conformational change is sufficient to enable Apaf-1 activation at the mitochondrial membrane (Jemmerson et al., 1999; Varkey et al., 1999).

Moreover, it was reported that efflux of cytochrome c could constitutes only a later event of the cell death, in other words, a side effect of the terminal dismantelment of the cell (Adachi et al., 1998; Krippner, et al., 1996). However, in this Fas-mediated apoptosis model, an inactivation of cytochrome c, correlated to the inhibition of mitochondrial respiration at the cytochrome c level, constitutes an very early causal event in the apoptotic program. It was suggested that inactivation of cytochrome c is associated to its release from the outer surface of the inner mitochondrial membrane where normally it functions as a shuttle connecting respiratory chain energy transducers (Skulachev, 1998a). More intriguing, the authors suggest that efflux of cytochrome c which had been associated with early events of apoptosis could be, in some instances, the result of a methodological artifact (Adachi, et al., 1998). Indeed, most studies are based on subcellular fractionations, i.e.: after cell disruption, centrifugation allows to separate soluble cytosolic elements in the supernatant from heavy membranes, including mitochondria, included in the pellet. In this approach, the in vivo cytochrome c redistribution is inferred from its appearance into the cytosolic fraction during the in vitro procedure. In fact, this method could be more properly considered as a measure of cytochrome c extractability from mitochondria rather than as a exact view of its in situ localization. For instance, some minor alterations of the outer mitochondrial membrane c could be compatible with a mitochondrial localization of cytochrome c in the cell and its outflow from mitochondria as fractionation provoked breakdown of the fragilized outer membrane. However, these considerations refute neither the early occurrence of cytochrome c release in various apoptotic models as observed by in situ immunolocalization and nor its crucial role in the apoptotic program, ascertained by direct microinjection of cytochrome c in the cytosol. They must be correlated to the evidenced existence of alternative pathways, i.e. cytochrome c independent, in the transduction of apoptogenic stimuli (Chauhan, et al., 1997; Li, et al., 1997a; Tang, et al., 1998).

The mechanism by which cytochrome c is released from mitochondria is largely unknown. The possible mechanisms involved in activation of this central control may be envisaged from data concerning mediators of the death signal transduction cascades.

For instance, the release of cytochrome c might result from oxidative imbalance, an upstream event in the apoptotic transduction cascade. These phenomena might lead to the alteration of some redox sensitive crucial regulatory elements of the outer mitochondrial membrane permeability, e.g. by a shift of the redox state of some sulfhydryl groups to a more inactivating oxidized state.

Another possibility comes from the ability of caspases to promote release intermembrane proteins, including cytochrome c, through the outer mitochondrial membrane. These observations

suggested that mitochondria carry a caspase substrate that, when cleaved, promotes cytochrome c-release (Mignotte and Vayssière, 1998). Recent reports concerning Fas and TNF-α receptor signaling could provide insight into how caspase induce cytochrome c efflux. Previously, it had been established that activation of caspases, predominantly the caspase-8, constitutes an early step in these apoptotic pathways (Nagata, 1997), and that inhibition of these proteases prevents both the release of cytochrome c from mitochondria and the execution of the cell death program (Schulze-Osthoff et al., 1998; Van der Heiden et al., 1997). Recently, it has been shown that caspase-8, activated by cell surface death receptors such as TNF and Fas, cleaves Bid, a death agonist of the Bcl-2 family, which in turn transduces the apoptotic signal from the cell surface to mitochondria (Li et al., 1998; Luo et al., 1998). Thus, while full-length Bid is localized in cytosol, the C-terminal part caspase-8-truncated BID translocates to mitochondria and then induces cytochrome c release, via a conformational change of Bax (Desagher et al., 1999) and the downstream caspase-dependent apoptotic program. Cytosolic substrates for effector caspases, other than Bid, could be involved in the induction of cytochrome c release (Bossy-Wetzel and Green, 1999). Overexpression of the pro-apoptotic Bcl-2 family member Bax in cells stimulates both the release of cytochrome c and apoptosis. By using isolated mitochondria and recombinant Bax, it was shown that Bax could directly induce cytochrome c release from mitochondria.

It has been shown that Bcl-2 and Bcl-X_L inhibit release of cytochrome c from the mitochondria into the cytosol (Gross, et al., 1999b; Kluck, et al., 1997a; Yang, et al., 1997). Thus, one level of action of Bcl-2 is to control the efflux of cytochrome c from the mitochondrial intermembrane space through the outer membrane and into the cytosol where caspases are found. It remains to be determined whether this efflux is done through Bax containing channels. It has recently been proposed that the cytochrome c released be regulated by the Bcl-2s members family, through the mitochondrial voltage-dependent anion channel (Shimizu et al., 1999). Post-mitochondrial fractions from cells that overexpress Bcl-2 both prevent and reverse cytochrome c inactivation in cell free experiments (Adachi et al., 1997), suggesting that Bcl-2 might even allow to transport cytochrome c back into mitochondria. Bcl-x_L inhibits AraC-induced preapoptotic accumulation of cytochrome c in the cytosol (Kim et al., 1997). Cells that overexpress Bcl-X_L fail to accumulate cytosolic cytochrome c or to undergo apoptosis in response to genotoxic stress. However, recent studies in the TNF-α death pathway indicate that, if Bcl-2 and Bcl-X_L are able to block cytochrome c release, other mitochondrial dysfunction and final cell death could nonetheless occur (Gross, et al., 1999b). Co-immunoprecipitation studies have shown that cytochrome c binds to Bcl-x_L and not to the proapoptotic Bcl-X_s protein, a pro-apoptotic derivative of Bcl-x_L. Thus, Bcl-X_s blocks binding of cytochrome c to Bcl-x_L (Kharbanda et al., 1997).

Even though, altogether, these results provide evidence that Bcl-2 family members can modulate cell death process by directly controlling the availability of cytochrome c in the cytosol. It must underlined that pro-survival actors, as Bcl-2/ Bcl-x_L, may also protect cells from apoptosis by inhibiting the availability of cytochrome c in the cytosol and act downstream of cytochrome c to prevent caspase activation under certain circumstances (Rosse et al., 1998; Zhivotovsky, et al., 1998). On the contrary, caspases inhibitors have no effect on this process.

These results point to a model where mitochondria might act as apoptotic amplifiers, fostering a positive feedback loop between cytochrome c-efflux and caspase activation. Any event that primes the loop will initiate the vicious "circle of death", leading to large-scale caspase activation and apoptotic cell death. However, this model does not prevail in all systems as, in numerous PCD, caspase inhibitors have no effect on the loss of cytochrome c (Bossy-Wetzel et al., 1998; Kluck, et al., 1997a; Kluck et al., 1997b).

4.2.2 AIF

The experiments described above showed that mitochondria from apoptotic cells can effectively control nuclear apoptosis and suggest the involvement of mitochondria derived products in the apoptotic cascade. Acellular experiments have permitted to show that mitochondria contain a pre-formed approximately 50-kD protein which is released upon PT pores opening and which causes isolated nuclei to undergo apoptotic changes such as chromatin condensation and internucleosomal DNA fragmentation. This protein, named AIF (Apoptosis-inducing factor), was initially described as a caspase-activating protein which promotes nuclear apoptosis in vitro probably via the activation of procaspase-3, a major executive caspase (Susin, et al., 1996). More recently, it has been shown that AIF could directly induce nuclear apoptosis, without any caspase activation (Susin et al., 1999b).

As expected, when preventing mitochondrial permeability transition, Bcl-2 overexpressed in the outer mitochondrial membrane also impedes the release of AIF from isolated mitochondria. In contrast, Bcl-2 does not affect the formation of AIF, which is contained in comparable quantities in control mitochondria and in mitochondria from Bcl-2-hyperexpressing cells. Furthermore, the presence of Bcl-2 in the nuclear membrane does not interfere with the action of AIF on the nucleus, nor does Bcl-2 overexpression protect cells against AIF. It thus appears that Bcl-2 prevents apoptosis by favoring the retention of an apoptogenic protein in mitochondria (Susin, et al., 1996).

The above-described AIF could be a key regulator of such a cytochrome c-independent pathway inasmuch as these two mitochondrial components seem involved in distinct signaling route. Indeed, while AIF release occurs after the PT-associated mitochondrial depolarization, several reports indicates that cytochrome c release is dissociable from PT (Bossy-Wetzel, et al., 1998; Jurgensmeier et al., 1998; Kluck, et al., 1997a; Li, et al., 1998; Van der Heiden, et al., 1997; Yang, et al., 1997). Furthermore, cytosolic cytochrome c requires additional cytosolic factors to promote apoptotic changes, via the activation of caspase-3, whereas AIF, once released from mitochondria, directly induces nuclear apoptosis without cytosol. Beyond their differences, these two "execution caspase" activating pathways illustrate the sophistication and the apparent molecular redundancy, which characterize the mammalian cell death. They may correspond to alternative and independent links between death-triggering stimuli and the execution machinery or, in contrast, they may work together to induce complete PCD (Golstein, 1997). Overexpression of Bcl-2/Bcl-x_L in cells or addition of recombinant Bcl-2 to cell-free systems containing mitochondria prevented cytochrome c/AIF's exodus from the mitochondria that was triggered normally by a wide variety of apoptogenic stimuli(Bossy-Wetzel, et al., 1998; Kharbanda, et al., 1997; Van der Heiden, et al., 1997; Yang, et al., 1997). The protective effect of Bcl-2 was linked to its ability to prevent the release of these proteins.

Alternatively, opening of PT pores, which is a common event of apoptosis, might be involved in AIF and cytochrome c outflow (Skulachev, 1998a). Opening of the PT pore could be a first step of a cascade of events causing an increase in matrix volume and subsequently a mechanic disruption of the outer membrane. Recent results show that this scenario is possible at least in vitro (Petit et al., 1998; Scarlett and Murphy, 1997; Van der Heiden, et al., 1997). Last, the pore-forming properties of Bax and other related proteins might suggest that they directly modulate the permeability of the outer mitochondrial membrane. Indeed, other studies have shown that Bax can induce cytochrome c release from mitochondria and caspase processing and activation (Jurgensmeier, et al., 1998). These events are sensitive to Bcl-x_L but caspase inhibitors have no effect on release of cytochrome c from mitochondria (Gross, et al., 1998; Xiang, et al., 1996) although they prevent the subsequent activation of caspases in cytosolic extracts. Unlike Ca^{++}, Bax did not induce PT and swelling of mitochondria in vitro in this system. These findings imply that Bax could uses an alternative mechanism for triggering release of cytochrome c from mitochondria may be through to the action of specific pores in the outer membrane.

5 Reactive oxygen species and apoptosis

5.1 What are ROS?

ROS, such as superoxide anion, radicals, hydrogen and organic peroxides are generated by all aerobic cells as by-products of a number of metabolite reactions and in response to various stimuli (Fridovich, 1978). Mitochondria are believed to be a major site of ROS production, according to an endogenous and continuous physiological process under aerobic conditions (figure 1). However, endoplasmic reticulum and nuclear membranes also contain e^- transport chains that can lose e^- and generate superoxide radical. Some fatty acid metabolites, such as those derived from arachidonic acid by the lipoxygenase pathway, are also ROS. The physiological activity of the respiratory chain leads to the production of semi quinones, a potential source of ROS (Papa and Skulachev, 1997).

Indeed, in aerobically respiring cells, the respiratory chain produces ROS at Complex I (NADH/Ubiquinone oxidoreductase) and Complex III (ubiquinol/cytochrome c oxido-reductase (Boveris et al., 1976; Takeshige and Minakami, 1979; Turrens et al., 1985). The ubiquinone site in complex III appears as the major site of mitochondrial ROS production: this site catalyzes the conversion of molecular oxygen to superoxide anion radical (O_2^-) by a single electron transfer to molecular oxygen (figure 1). Moreover, the inhibition of the respiratory chain, due to a lack of oxygen or to an inhibitor, such as cyanide or antimycin A, increases the ubisemiquinone free radical level in the normal catalytic mechanism of complex III (Turrens, et al., 1985).

In aqueous solutions, O_2^- is moderately reactive but can generate other potent ROS such as H_2O_2 by dismutation. About 1-5% of mitochondria O_2 consumption leads to H_2O_2 production (Chance et al., 1979). A reductive homolytic cleavage of H_2O_2 produces the highly oxidative and cytotoxic hydroxyl radical ($OH^{\cdot}$) according a metal-dependent breakdown. This highly reactive hydroxyl radical, by-product of superoxide anion or hydrogen peroxide, is assumed to be directly responsible for most of the oxidative damages leading to the non-physiological necrosis (Halliwell and Gutteridge, 1990). Alternatively, mitochondria superoxide may react with nitric oxide to produce peroxynitrite (Groves, 1999), a potent oxidant, which causes irreversible inhibition of mitochondria respiration and damage to mitochondria components (complexes I, II, IV and V, creatine kinase, aconitase, membranes, DNA, SOD…). It induces cell death in several cell types (Lin et al., 1998; Liu et al., 1999; O'Connor et al., 1997; Szabo and Ohshima, 1997). Brown recently reviewed the relationship between nitric oxide and mitochondria respiration (Brown, 1999).

5.2 Oxidative stress

An excess of reactive oxygen free radical species (ROS) in mitochondria causes an oxidative stress, with an enhanced activity of the antioxidant defense system and mitochondrial damage. Several situations, such as dysfunctional complex I (Robinson, 1998), chemical poisoning and ischaemia followed by reperfusion, may subject tissues and cells to oxidative stress The main targets of ROS in mitochondria are the protein components of the membranes and the polyunsaturated fatty acids. In membranes, ROS and lipids affect cysteine residues (sulfhydryl groups), causing intramolecular cross-linkings and formation of proteins aggregates. $OH^{\cdot}$ radicals can initiate lipids peroxidation and generate peroxyl- and alkoxyl radical intermediates. Oxidants increase the release of calcium from mitochondria, thus stimulating calcium-dependent enzymes, such as proteases, nucleases, phospholipases involved in apoptosis. Because of the absence of

mitochondria DNA-protecting proteins, the low efficiency reparation mechanism and the proximity of the respiration chain, mtDNA is a privileged target for ROS (Shoji et al., 1995).

5.3 Protection of the cell

To prevent oxidative damage, mammalian cells have developed a complex antioxidant defense system that includes non-enzymatic antioxidants (e.g. glutathione, thioredoxine) as well as enzymatic activities (e.g. catalase, superoxide dismutase (SOD)) (Sies, 1991).
Mitochondria possess an antioxidant system with the superoxide dismutase (SOD), NADH and a complete glutathione redox system, which is formed of glutathione reductase, reduced glutathione (GSH), and glutathione peroxidase (figure 1). This system allows the reduction of oxidants, such as hydroperoxide. As mitochondria are generally devoid of catalase, the hydroperoxide detoxification mainly relies on GSH peroxidase. Various chemicals are known to induce oxidative stress, either as the result of an enhanced free radical production (e.g. xenobiotics utilizing cytochrome P450 for detoxification) or by impairment of their destruction provoked by glutathione depletion. Reed has shown that irreversible injury may occur if the mitochondria pool of glutathione is depleted by about 50% by incubation conditions or any toxic agent (Reed, 1990). Mitochondrial GSH plays an important role in preserving mitochondria membrane integrity and in assuring the reduced state of the intramitochondrial protein thiol groups.

Under unfavorable conditions, such as excess of ROS, mitochondria can enlarge and form "megamitochondria" with lower membrane potential and decreased rate of oxygen consumption and ROS production (Karbowski et al., 1999). Incubation of adriamycin- or hydrazin- treated rat hepatocytes with exogenous coenzyme Q10 suppresses the formation of megamitochondria and inhibits the increase of hydrogen peroxide and the drop of mitochondria potential, normally found in these treatments. (Lenaz et al., 1998; Teranishi et al., 1999). Thus, the antioxidant effect of quinone acts on the membrane potential and the rate of generation of ROS.

Furthermore, Skulachev proposed that a mitochondrial mild uncoupling and non-coupled oxidations could safe guard cells from high oxygen level and ROS generation (Skulachev, 1996a; Skulachev, 1996b; Skulachev, 1998b).

5.4 Physiological role

ROS play a role in physiological systems: they were shown to be responsible for the inducible expression of genes associated with inflammatory and immune responses. Current evidence indicates that different stimuli use ROS as signaling messengers to activate transcription factors, such as AP-1 and NF-κB, and induce gene expression (Pinkus et al., 1996). Additionally, ROS may have divergent effects according to the cell type, as they can function as mitotic stimuli (Clement and Pervaiz, 1999), inhibitors of drug-induced tumor cell death (Pervaiz et al., 1999) or as cell death mediators (Burdon, 1995; Burdon, 1996). Indeed, superoxide mediates or induces proliferation of cardiac (Li et al., 1999), vascular smooth muscle cells (Bhunia et al., 1997; Brown et al., 1999; Lee et al., 1998; Li et al., 1997c; Rao and Berk, 1992). On the opposite, it induces cell death in cardiomyocytes (Li, et al., 1999), hippocampal neurons (Krohn et al., 1998) or proximal tubular cells (Lieberthal et al., 1998)
The ability of oxidative stress, which is an excessive production of ROS, to provoke necrotic cell death as a result of massive cellular damages associated to lipid peroxidation and alterations of proteins and nucleic acids, is well documented for a long time (Halliwell and Gutteridge, 1989). When free radicals generation exceeds the cellular capacity to detoxify them, cellular protein and

DNA damage may occur, leading to the cell death In this point of view, aerobic cells appear as being under a continual "oxidative siege", their survival depending on a balance between ROS and antioxidants.

The possible implication of ROS as signaling molecules in more physiological deaths such as apoptosis is a more recent concept. First evidence suggesting the involvement of mitochondria in cell death arose from the study of the TNF-α-induced cytotoxicity (Lancaster et al., 1989; Schulze-Osthoff et al., 1992). In addition to their role during the effector phase of apoptosis, an alteration of the mitochondrial function was associated with the early phases of the cell death and was defined as a crucial step of the process. The observed inhibition of the mitochondrial respiratory chain was assumed to result in the over production of Reactive Oxygen Species (ROS) which would act as mediators of the death signaling pathway (Schulze-Osthoff et al., 1993). Thus, there is mounting evidence that these compounds may be central in the cell death transduction pathways.

ROS are important physiological reactants in mitochondria (Richter et al., 1995) and several observations suggest that ROS might mediate PCD:

1) The addition of ROS or the depletion of endogenous antioxidants can promote cell death (Carmody et al., 1999; Guénal, et al., 1997; Kane et al., 1993; Lennon et al., 1991; Ratan et al., 1994; Sato et al., 1995).

2) Some antioxidants, such as thioredoxine or N-acetylcystein, act as intracellular ROS scavengers, and can inhibit activation of caspases and subsequent steps leading to the apoptotic cell death. In these models, ROS could be considered as major mediators, and not only the source of direct damage to DNA, proteins and lipids (Greenlund et al., 1995; Iwata et al., 1997; Mayer and Noble, 1994; Mehlen et al., 1996; Sandstrom and Buttke, 1993; Wong et al., 1989).

3) Increases in intracellular ROS are sometimes associated with PCD (Li, et al., 1999; Manna et al., 1998; Martin and Cotter, 1991; Quillet-Mary et al., 1997; Uckun et al., 1992)

4) MnSOD overexpression restores mitochondrial transmembrane potential (Majima et al., 1998) and inhibits the cell death caused by an inhibition of the respiratory chain (Kiningham et al., 1999)

5) Cu/Zn SOD overexpression delays apoptosis by scavenging of O_2- in neuronal cells deprived of nerve growth factor (Greenlund, et al., 1995)

6) Mitochondrial phospholipid hydroperoxide glutathione peroxidase overexpression inhibits increase of ROS, release of cytc, activation of caspase-3 and apoptosis (Nomura et al., 1999)

ROS could be considered as major mediators, and not only the source of direct damage to DNA, proteins and lipids (Tan et al., 1998)

5.5 ROS participate in early and late steps of the regulation of apoptosis

In addition to their role in TNF-α-induced killing (figure 2), the contribution of ROS to the activation of the execution machinery was extended to PCD triggered by a wide range of influences including UV light, ionizing irradiation, anthracyclines, ceramides, glucocorticoids or survival-factor withdrawal (Jacobson, 1996). In different models, the ROS generation preceded the loss of mitochondria membrane potential, nuclear condensation and other typical apoptotic events. For example, an early and sustained production of ROS with a concomitant depletion of intra cellular glutathione was observed in an *in vitro* model of photoreceptor apoptosis (Carmody, et al., 1999). Increase in ROS levels is an early process in Fas-induced apoptosis, independent from caspases activation (Banki et al., 1999). In ischaemia-induced apoptosis, ROS are involved upstream of caspases and bax (Maulik et al., 1998). Tada-Oikawa et al. showed that during DNA alkylation-

induced apoptosis, the hydrogen peroxide generation acted upstream the mitochondria membrane potential drop (Tada-Oikawa et al., 1999).

However, in K+ deprivation induced apoptosis of cerebellar granule neurons, ROS accumulation plays an important role during the late steps. In this model, the ROS production is prevented by cycloheximide, actinomycin D and caspase inhibitor YVAD-cmk, indicating that ROS are involved downstream of gene transcription, traduction, and activation of caspases (Schulz et al., 1997; Schulz et al., 1996).
Moreover some data raise the possibility that ROS are also required for the execution of the death program (Kroemer, et al., 1995). This must be cautiously considered inasmuch as, in the majority of these systems, it is difficult to ascertain that the observed ROS accumulation corresponds to a causal effect and is not a side effect of the other changes accompanying the killing process (Cai and Jones, 1998). Moreover, in these cases, ROS increase most often arises during the later stage of the death program, i.e. during the destruction phase when the cell is broken down, and may be associated with a necrotic type terminal degradation of the cell. Exogenous sources of ROS such as hydrogen peroxide can induce PCD or necrosis depending upon the dose added (Guénal, et al., 1997). So a burst in ROS, in response to a dramatic perturbation of the physiology of the dying cell, could convert the late PCD steps into necrotic death. Therefore, it appears that at any moment the level of intracellular ROS can determine the fate of the cell: low levels of ROS can induce PCD while accumulation of high levels promotes necrosis or can lead PCD-committed cells toward necrotic-like destruction.

5.6 Origin of apoptosis-mediated ROS

Two opposite models have emerged concerning the source of signaling ROS, in relation with the variety of metabolic reactions and intracellular sites which can generate ROS (Jacobson, 1996). While most investigators believe that oxidants are produced by electron chain transport, some data seem to moderate this point of view. Fatty acid metabolites, such as those produced from arachidonic acid by the lipoxygenase pathway, may be better mediators of apoptosis (O'Donnell et al., 1995). On the one hand, it is argued that these molecules harbor a more specific reactivity than superoxide anion and its by-products, this biological specificity being assumed necessary for a signaling role in apoptosis transduction pathways. On the other hand, it was shown that exogenous fatty acid metabolites can promote programmed cell death and that, in some cases, their increased production was associated with cell death. It must be underlined that such a situation is limited to systems where the death signal result is mediated by surface receptors. Nevertheless, these considerations do not refute the compelling evidence of the involvement of electron transport chain-produced ROS in cell death signaling. It appears more reasonable to consider that, depending upon the cell death stimulus and the cell model, these two types of ROS can mediate apoptosis or even both contribute to the activation of the execution machinery, as suggested by studies of TNF-α-induced programmed cell death.

It was established that both ROS accumulation and programmed cell death process require the presence of a functional mitochondrial respiratory chain in most ROS-dependent cell death systems (Higuchi et al., 1997; Quillet-Mary, et al., 1997; Schulze-Osthoff, et al., 1993; Sidoti-de Fraisse et al., 1998). Indeed, it was shown that an upstream inhibition, with chemical compounds acting on complex I (Quillet-Mary, et al., 1997; Schulze-Osthoff, et al., 1992), or an elimination of the electron transfer chain (Higuchi, et al., 1997; Schulze-Osthoff, et al., 1993; Sidoti-de Fraisse, et al., 1998), by depletion of the mtDNA, prevent ROS accumulation and consequently protect cells against PCD. Another indirect argument is provided by the scavenger role of mitochondrial glutathione in the regulation of ROS-mediated PCD (Goossens et al., 1995). The ubiquinone site in complex III appears as the major site of mitochondrial ROS production as this site catalyzes the

conversion of molecular oxygen to superoxide anion which can lead to the formation of other potent ROS such as hydrogen peroxide and hydrogen radicals (figure 1). The inhibition of the respiratory chain, due to a lack of oxygen or to an inhibitor, such as cyanide or antimycin A, increases the ubisemiquinone free radical level in the normal catalytic mechanism of complex III (Turrens, et al., 1985). Such a model is supported by the observed potentiation of cell death processes in ROS-dependent PCD when electron flow was inhibited distal to the ubiquinone pool.

5.7 Mechanisms of ROS signalling

The involvement of mitochondrial ROS in some cell death transduction pathways next leads to the fundamental questions concerning, on the one hand, the causal event of the increased ROS generation and, on the other hand, the molecular mechanisms underlying the ROS signaling. Two viewpoints must first be considered to address the question of the origin of ROS accumulation, which can indeed result from an increased production or from a reduced scavenging by the cellular detoxifying systems. Much of the available data converge to the hypothesis that ROS increases are the consequence of an impairment of the mitochondrial respiratory chain (Cai and Jones, 1998; Degli Esposti and McLennan, 1998; France-Lanord et al., 1997; Gudz et al., 1997; Quillet-Mary, et al., 1997; Schulze-Osthoff, et al., 1992). In agreement with the above considerations, the observed alterations are distal to the ubiquinone site of the complex III, but the origin of these electron flow disturbances are not clear. The only strong evidence comes from the study of ceramide-induced PCD, in which an increased H_2O_2 production was linked to mitochondrial Ca^{2+} homeostasis perturbation as inhibition of the mitochondrial Ca^{2+} uptake was shown to abolish both ROS accumulation and cell death. However, the recently observed shift of cytochrome c from mitochondria to cytosol in the early phases of many PCD (see above) could provide a clue to resolve this question (Kluck, et al., 1997a; Liu, et al., 1996; Yang, et al., 1997). Indeed, the release of cytochrome c must lead to a breakdown of the mitochondrial electron flow downstream of the ubiquinone site, which in turn would result in an increased generation of ROS. Such a model is supported by the described correlation between loss of cytochrome c activity and respiratory failure in a Fas-induced PCD model (Krippner, et al., 1996) and by the study of mitochondria isolated from apoptotic cells which shows a superoxide production due to a switch from the normal 4-electron reduction of O_2 to a 1-electron reduction when cytochrome c is released from mitochondria (Cai and Jones, 1998).

Beside the question of the process of mitochondrial ROS accumulation, arises the problematic concerning the targets of these compounds or more precisely: how can they mediate PCD? Two models can be proposed to approach this conflicting and not well-documented subject. The first model assumes that ROS themselves are signaling molecules, which activate some crucial components of the PCD machinery. Conversely, the alternative proposition suggests that ROS can act indirectly by modifying the cellular redox potential, which would regulate some key regulatory proteins involved in PCD. Several lines of evidence agree with an explanation based on indirectly mediated action. First, unlike fatty acid metabolites which harbor specific reactivity and are known to mediate particular signals from surface receptors, mitochondrial ROS are characterized by a lack of biological specificity or even an extreme reactivity, as for the hydroxyl radical: these are all features contrary to the requirements of a specific signaling role (Jacobson, 1996). In this way, a direct influence of ROS on PCD process would be correlated to a general damaging effect on cellular structures resulting in necrotic cell death, or perhaps to a more limited action on mitochondria, their site of production, which in turn could activate some mitochondria-dependent downstream cascades leading to PCD. Secondly, despite the compelling evidence of the role of mitochondrial ROS in PCD signaling pathways, it has been assumed that they do not represent a general mediator of cell death, as suggested by the ability of some PCD to occur in very low oxygen

environments (Jacobson and Raff, 1995; Shimizu et al., 1995). However, ROS might be produced in such conditions (Degli Esposti and McLennan, 1998).

Another alternative explanation would be to consider that the major effect of an increased ROS production is the subsequent imbalance of intracellular redox status, i.e. an enhancement of the cellular oxidative tonus, and that in fact the oxidative stress is the central common effector of PCD. Hence, ROS accumulation would only be one way which leads in some PCD to an oxidative status. Anti-Fas/APO-1 antibody or IL-3 withdrawal-induced PCD represent good illustrations of this model (Bojes et al., 1997; van den Dobbelsteen et al., 1996). Indeed, no ROS accumulation can be measured in these two systems and anaerobic cultured cells deprived of IL-3 still undergo PCD (Schulze-Osthoff et al., 1994a; Shimizu, et al., 1995). However, an oxidative stress can be shown in these models as a depletion of glutathione (GSH), a non-enzymatic cellular antioxidant, as a result of a rapid and specific efflux of glutathione, an event that takes place at the very beginning of the apoptotic process (Bojes, et al., 1997; van den Dobbelsteen, et al., 1996). Moreover, it has been shown that Bcl-2 can protect cells from PCD by shifting the cellular redox potential to a more reduced state (see below). However, the observation that oxidation of thiols other than glutathione can mediate induction of PCD suggest that the intracellular thiol redox status would be the real key factor of the cell death signaling pathways (Kane, et al., 1993; Marchetti et al., 1997; Mirkovic et al., 1997; Sato, et al., 1995). In this model, the redox state of glutathione or other cellular antioxidants such as thioredoxine, would be in equilibrium with that of thiols resident in some redox sensitive crucial components of the execution machinery (Kroemer, et al., 1997). Where does ROS fit this thiol hypothesis? In this putative model, an increased production of mitochondrial ROS would result, either by a direct modification of the thiols or indirectly via a depletion of the intracellular antioxidant pool, in a shift of the redox state of the sensor SH groups to a more oxidized state. The nature of the ROS and the level of the intracellular antioxidant defenses would determine in which way regulatory components are activated to commit cells to PCD. In a hypothetical model, mitochondrial ROS modify the membrane permeability leading to the release of pro-apoptotic factors and/or activate directly executing caspases.

5.8 Bcl-2 and Bcl-X_L inhibits apoptosis by providing a protection against ROS and/or shifting the cellular redox potential to a more reduced state

Several lines of evidence support the idea that Bcl-2 acts in an antioxidant pathway to suppress apoptosis. Yeast mutants lacking superoxide dismutase were partially rescued by expression of Bcl-2 (Kane, et al., 1993; Longo et al., 1997). Oxidative stress, such as exposure to hydrogen peroxide or 13-L-hydroperoxylinoleic acid, increases the level of ROS and triggers loss of $\Delta\Psi m$, apoptotic nuclear condensation and DNA fragmentation in normal PC12 cells. When transfected in these cells, Bcl-2 prevents the decrease in $\Delta\Psi m$ and the cell death induced by oxidative stress but not the increase in level of (Satoh et al., 1997). This indicates that Bcl-2 acts downstream of ROS generation in this system.

Following an apoptotic signal, overexpression of Bcl-2 suppressed lipid peroxidation completely (Hockenbery et al., 1993) and attenuates the generation of ROS (Bogdanov et al., 1999). Bcl-2 deficient mice turn gray with the second hair follicle cycle, implicating possibly a defect in redox-regulated melanin synthesis (Veis et al., 1993). Bcl-2 can protect neural cells from delayed death resulting from chemical hypoxia and reenergization, and may do so by an antioxidant mechanism (Myers et al., 1995) (reviewed in (Korsmeyer et al., 1995).

It was shown that Bcl-2 might act as an antioxidant partner to block a putative ROS-mediated step in the cascade of events required for apoptosis, but the way by which Bcl-2 protects from ROS remains unclear. In some systems, Bcl-2 appears to influence the generation of oxygen

free radicals (Kane, et al., 1993), while in other cases it does not affect ROS production but does prevent oxidative damage to cellular constituents (Hockenbery, et al., 1993; Satoh, et al., 1997; Tyurina et al., 1997). Bcl-2 acts locally in mitochondria and plasma membranes, as assessed by electron paramagnetic resonance spectroscopy analysis (Bruce-Keller et al., 1999). Alternatively, since superoxide is produced by mitochondria from apoptotic cells due to a switch from the normal 4-electron reduction of O_2 to a 1-electron reduction, the block of cytochrome c release could provides a mechanism for the apparent antioxidant function of Bcl-2 (Cai and Jones, 1998). It has also been proposed that it functions as a pro-oxidant and influences the levels of ROS, inducing in this way endogenous cellular antioxidants (Steinman, 1995).

However, apoptosis can proceed normally, and can be prevented by Bcl-2, under anaerobic conditions which minimize the formation of ROS (Jacobson and Raff, 1995; Shimizu, et al., 1995). This observation reinforces the view that Bcl-2 acts in more than one way either to prevent the induction of apoptosis by different stimuli (ROS dependent or not) or to control different aspects of the apoptotic effector pathway (reviewed in (Jacobson, 1996). As described above, apoptosis might be modulated by redox sensitive proteins via their sulfhydryl groups and antioxidants such as reduced glutathione or other thiols may modify the functions of these proteins.

Several authors have studied the effect of Bcl-2s on cellular redox potential. Activities of antioxidant enzymes and levels of glutathione and pyridine nucleotides have been measured in pheochromocytoma PC12 and the hypothalamic GnRH cell line GT1-7 cells transfected with bcl-2 (Ellerby et al., 1996) Both cell lines overexpressing bcl-2 had elevated total glutathione levels when compared with control transfectants. The ratios of oxidized glutathione to total glutathione in PC12 and GT1-7 cells overexpressing bcl-2 were significantly reduced. In addition, the NAD+/NADH ratio of bcl-2-expressing PC12 and GT1-7 cells was two- to threefold less than that of control cell lines while they had approximately the same level of catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase activities as control cells. These results indicate that the overexpression of bcl-2 shifts the cellular redox potential to a more reduced state, without consistently affecting the major cellular antioxidant enzymes. Furthermore, depleting cellular thiols reversed the resistance to radiation in Bcl-2 expressing lymphoma cell lines (Mirkovic, et al., 1997).

The ability of bax and Bcl-x_L to affect GSH was assessed in interleukin (IL)-3-dependent murine prolymphocytic FL5.12 cells (Bojes, et al., 1997) Overall levels of GSH increased in bcl-x_L transfectants while, in cells overexpressing bax, GSH was reduced by approximately 36%. There were no consistent differences between these cell lines in the activities of superoxide dismutase, catalase, and glutathione peroxidase or glutathione reductase. Following IL-3 withdrawal-induced apoptosis, control cells and bax transfectants exhibit a rapid loss of intracellular GSH that seemed to occur due to a translocation out of the cell. Cells overexpressing bcl-x_L did not lose significant amounts of GSH upon withdrawal of IL-3, and no apoptosis was evident. These results suggest a possible role for GSH in the mechanism by which Bcl-x_L prevents cell death.

Thus, both Bcl-2 and Bcl-x_L can protect cells from apoptosis by shifting the cellular redox potential to a more reduced state. Assuming that mitochondrial thiols constitute a critical sensor of the cellular redox potential during apoptosis (Marchetti, et al., 1997), these effects could be at the mitochondrial level.

6 Yeast and apoptosis

Another exciting question to resolve is the occurrence of an apoptosis-like phenotype associated with a specific mutation in *Saccharomyces cerevisiae* (Madeo et al., 1997). Indeed, the $cdc48^{S565G}$ mutant show typical markers of apoptosis, including exposure of phosphatidylserine, chromatin condensation and fragmentation, DNA fragmentation, and formation of minicells, which look like apoptotic bodies. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* yeast species do not contain endogenous caspases or Bcl-2s gene (Greenhalf et al., 1996; Ink et al., 1997; Zha et al., 1996b) and their cytochrome c has been shown to be ineffective to induce nuclear

apoptosis in acellular assay (Kluck, et al., 1997b). Moreover, if caspases are important in the mammalian apoptotic process, some models reveal a caspase-independent pathway. For example, Bax or Bak expression, and cytosolic AIF induce apoptosis in presence of broad caspase inhibitors (McCarthy et al., 1997; Monney et al., 1998; Susin, et al., 1999b; Xiang, et al., 1996). The data in yeast suggest the existence of yet unknown PCD pathways, independent of Bcl-2s and caspases families, in which the place and the role of mitochondria remain to be determined

The heterologous expression of mammalian pro-apoptotic genes (ced-4, bax, bak) provokes mitochondrial dysfunction and the death of yeast cells (Greenhalf, et al., 1996; Hanada et al., 1995; Ink, et al., 1997; James et al., 1997; Ligr et al., 1998; Sato et al., 1994). Several authors reported that this induced-cytotoxicity was characterized by typical phenotypic markers of apoptosis (Ink, et al., 1997; Ligr, et al., 1998), identical to those observed in the pseudo-apoptotic mutant $cdc48^{S565G}$ (Madeo, et al., 1997), and other features, such as release of cytochrome c (Manon et al., 1997). Moreover, as observed in mammals, coexpression of Bcl-2 or Bcl-X_L with Bax abolishes the pseudo-apoptotic phenotype and the cell death (Jürgensmeier et al., 1997; Ligr, et al., 1998; Manon, et al., 1997; Matsuyama et al., 1998; Tao et al., 1997; Zha et al., 1996a). Since on the one hand, Bax cytotoxicity is increased under respiratory condition (Priault et al., 1999), and, on the other hand, yeast mutants lacking superoxide dismutase were partially rescued by expression of Bcl-2 (Kane, et al., 1993; Longo, et al., 1997), it is tempting to speculate that a mitochondrial ROS production is involved in the bax-induced cell death process. In agreement with this hypothesis, the apoptotic-like features observed in these yeast strains is accompanied with ROS accumulation in the cells (Madeo et al., 1999). Moreover, low concentration of H_2O_2 treatment or glutathione depletion could induce the pseudo-apoptosis in yeast (Madeo, et al., 1999). The induced-cell death is not due to a direct cell damage, but requires an active cooperation of the cell machinery. ROS accumulation in cells seem to be necessary and sufficient to lead to the pseudo-apoptotic phenotype in yeast, whatever the apoptosis inducer.

7 Conclusion

In conclusion, mitochondria are involved in the decision of cells to survive or not at several levels. At a first level, mitochondria are involved in the control of the activation of the cell death machinery by sequestering, via Bcl-2 family proteins, pro-caspases and caspase activators, such as AIF or cytochrome c. However, the mechanism allowing the release of AIF and cytochrome c needs further investigations. Opening of the PT pore could be just a first step of a cascade of events causing an increase in the permeability of the outer mitochondrial membrane, and Bcl-2 could inhibit apoptosis by preventing permeability transition. At present, it remains elusive whether this permeability increase is due to the action of specific pores in the outer membrane and/or to its mechanic disruption secondary to an increase in matrix volume.

This recent accumulation of data has also generated a number of new questions. Future works must address the connection between survival signals and mitochondrial functions. The study of Bad regulation, a proapoptotic Bcl-2 member that is supposed to counteract Bcl-2 has provided an example of how survival factors counteract PCD. Indeed, Bad is inactivated by phosphorylation by Akt (Datta et al., 1997; del Peso et al., 1997) and Raf-1 (Wang et al., 1996), two kinases involved in survival signals transduction. It has been suggested that, in the absence of phosphorylation, Bad induces cell death possibly via the formation of heterodimers with Bcl-x_L (or Bcl-2 depending of the cell type) and the concomitant generation of Bax homodimers. Assuming that all mammalian cells constitutively express all the protein components required to execute the death program (Jacobson et al., 1994), these results suggest that Bax (or an other similar pro-

apoptotic member of the Bcl-2 family) are ubiquitously expressed and that survival requires their continuous inhibition. Taking into account the mitochondrial membrane localization of these proteins and their pore forming properties, it can be proposed that this kind of regulation operates at the mitochondrial level to control membrane permeability and efflux of AIF and cytochrome c.

The importance of mitochondria in apoptosis has been reinforced by studies showing the contribution of reactive oxygen species in the cell-death signaling. The mechanism of ROS signaling is still unknown, but it could be speculated that they act by modifying Bcl-2 proteins family conformation. Indeed, it was proposed that in healthy cells Bax is a cytosolic soluble monomer, whereas upon a death stimulus the Bax COOH-terminal hydrophobic domain becomes exposed, allows Bax redistribution to organelles, promoting cell death (Hsu and Youle, 1998; Wolter, et al., 1997). One could imagine that such a conformational change is induced by ROS. If the primer function of apoptosis was possibly to clear ROS-producing cells from tissues (Skulachev, 1996b; Skulachev, 1998a), mitochondrial ROS production are used as active mediators in the regulation of apoptosis at different levels. Moreover, the fact that pseudo-apoptosis phenotype in yeast invariably involves ROS (Madeo, et al., 1999) points out these compounds as major evolution-conserved regulators of cell death.

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Figure 1: Schematic representation of the formation and the fate of superoxide ions ($O_2^{\cdot -}$) in mitochondria.

ROS can be generated by various enzymes (xanthine oxidase, lipoxygenases, NADPH oxidases,…) or during the mitochondrial oxidative phosphorylation at complex I and complex III (see text). Complex I (NADH-ubiquinone oxidoreductase) catalyzes the following reaction with electron transfer : $NADH + Q + nH^+_{(matrix)} \rightarrow NAD^+ + QH_2 + (n-1)H^+_{(intermembrane\ space)}$, where Q is the ubiquinone Q10. The ubiquinone site (UQ) in complex III catalyzes the conversion of molecular oxygen to superoxide anion radical ($O_2^{\cdot -}$) by a single electron transfer to molecular oxygen. $O_2^{\cdot -}$ are converted to hydrogen peroxide (H_2O_2) by the superoxide dismutase (SOD).

H_2O_2 detoxification normally occurs according two different reactions, either by the thiol-specific antioxidant and the thioredoxine reductase (TR), which reduces thioredoxine (TX -> TX-H_2), or by reacting with glutathione (GSH) by glutathione peroxidase (GPO). The latter reaction produces water and oxidized glutathione (GSSG), and GSSG is recycled to GSH by glutathione reductase (GR). Glutathione is mainly maintained in a reduced state, via NADPH generation in the reversible non-oxidative part of the pentose phosphate pathway (Banki et al., 1996). When GSH level is low, H_2O_2 can react with Fe^{2+} to produce OH^- and the harmful hydroxyl anions ($OH^{\cdot -}$). Alternatively, H_2O_2 reacts with $O_2^{\cdot -}$ producing water, molecular oxygen and $OH^{\cdot -}$.

Superoxide may also react with nitric oxide (NO) to produce peroxynitrite ($ONOO^{\cdot -}$), a potent oxidant, which causes irreversible inhibition of mitochondria respiration and damage to mitochondria components (complexes I, II, IV and V, creatine kinase, aconitase, membranes, DNA, SOD,…) and induce cell death in several cell types.

If ROS production exceeds the cellular ability of detoxification (oxidative stress), matrix and membrane proteins, DNA and/or lipids can be damaged (▬ · · ▬).

The complexes of the electron transfer chain in the inner mitochondrial membrane are numbered from I to IV. The electron (e-) pathway through the respiratory chain is indicated by arrows. Cyt c refers to cytochrome c.

Figure 2. ***Schematic model of the role of mitochondrial ROS in apoptosis***

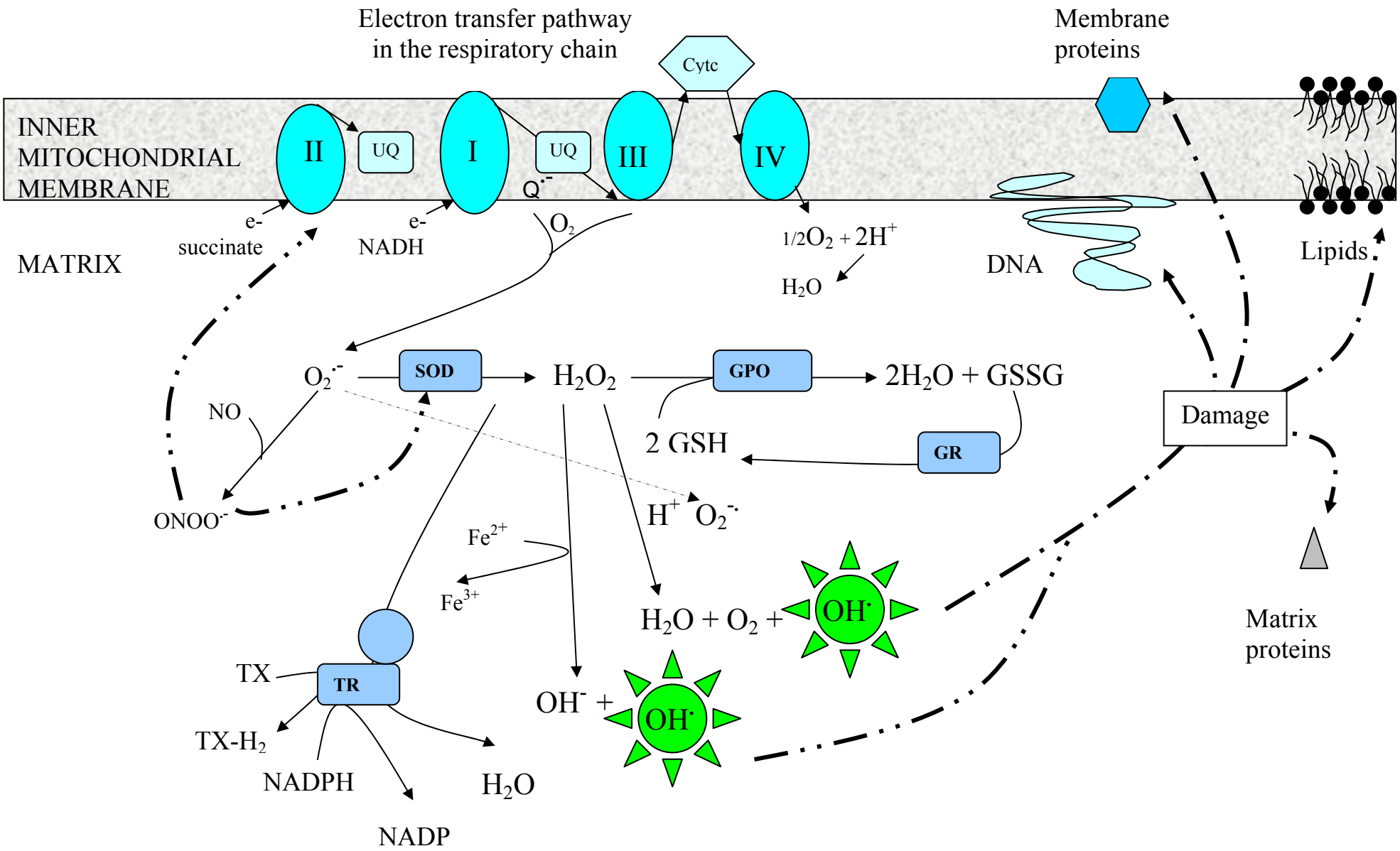
Numerous signals can lead to apoptosis and the induction pathways seem to converge to events involving mitochondria. Alternatively, activating caspases directly activate the executing caspases in a mitochondria-independent manner (□). At a first level, mitochondria can contribute to apoptosis signaling, as shown in TNF-α- or ceramide-induced cell death, during which increased mitochondrial ROS production appears as an early event of the induction phase. ROS accumulate as a result of a dysfunction in the mitochondrial respiratory chain. ROS also intervene in later steps in an amplifying loop, acting on mitochondrial membrane permeability, and contributing then to the activation of execution caspase.

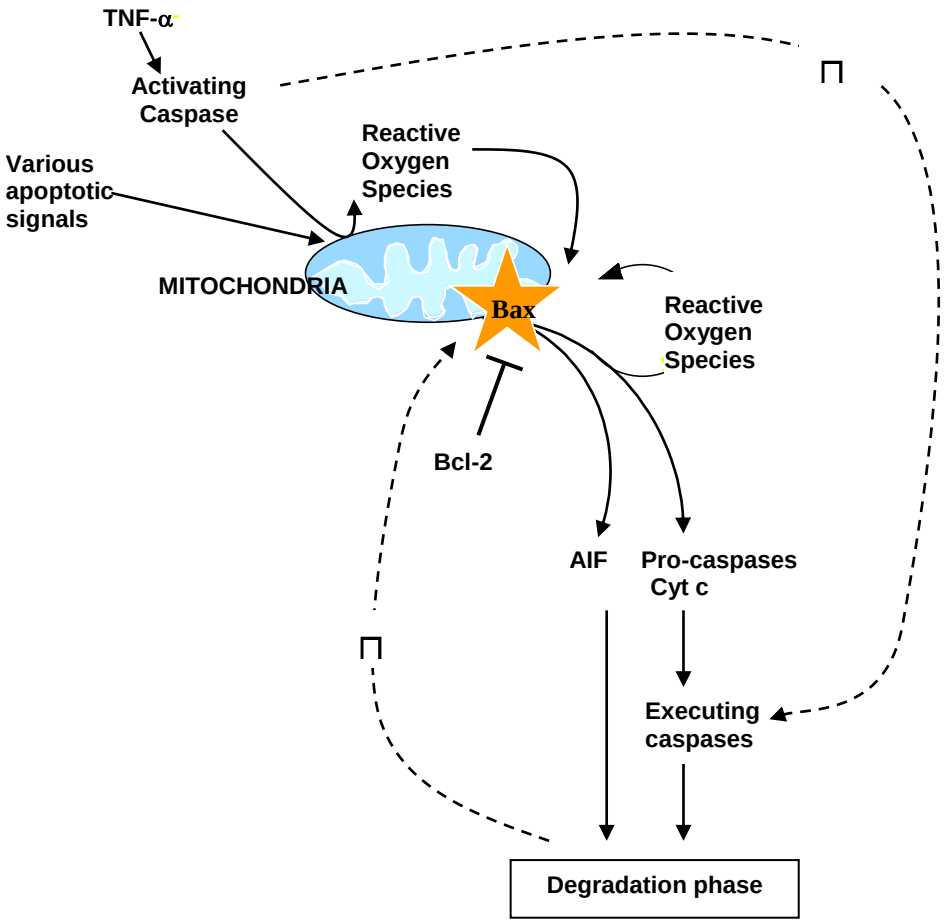
Proteins of the Bcl-2 family act on mitochondrial membrane permeability and regulate the release of pro-apoptotic factors from the intermembrane space to the cytosol. These apoptogenic factors activate proteins involved in the final degradation of the cell components, in executing caspases -dependent (procaspases and cytochrome c) or –independent (AIF) pathways. In return, they can act in an amplification loop upon mitochondria (□)

Table I: Programmed cell death in nematodes and mammals is controlled by homologous proteins.

Mammalian caspases act either during the activation or the execution phase of PCD (Nicholson and Thornberry, 1997). A CED-4 homologue has been identified in human cells (Hofmann et al., 1997; Zou, et al., 1997) In mammals some members of the Bcl-2 related proteins are death antagonists while other are death agonists. For a recent review on the structure-function relations of Bcl-2 related proteins see (Gross, et al., 1999a; Kroemer, 1997).

C. elegans	Mammals	Putative function of proteins
ced-3	*Activating caspases*: caspase-1 (ICE), -4 (ICH-2), -6 (Mch2), -8 (MACH/FLICE)… *Executing caspases*: caspase-2 (ICH-1), -3 (CPP32), -4 (ICH-2), -7 (ICE-LAP3)…	Caspase (cysteinyl aspartase)
ced-4	*apaf-1*	Caspases activator
ced-9	*Anti-apoptotic*: Bcl-2, Bcl-x_L, Bcl-w, Bfl-1, Brag-1, Mcl-1, A1, NR13… *Pro-apoptotic*: Bax, Bak, Bcl-x_S, Hrk…	Regulators of the release from mitochondria of apoptogenic factors
egl-1	Bik, Bad, Bid…	Inhibitors of antiapoptotic proteins of the Ced-9/Bcl-2 family


Electron transfer pathway
in the respiratory chain
Membrane
proteins
Cytc
INNER
MITOCHONDRIAL
MEMBRANE
II
UQ
I
UQ
III
IV
e-
succinate
e-
NADH
$Q^{\cdot -}$
O_2
$1/2O_2 + 2H^+$
H_2O
MATRIX
DNA
Lipids
$O_2^{\cdot -}$
SOD
H_2O_2
GPO
$2H_2O + GSSG$
NO
2 GSH
GR
Damage
$ONOO^-$
H^+ $O_2^{\cdot -}$
Fe^{2+}
Fe^{3+}
$H_2O + O_2 +$
$OH^{\cdot}$
Matrix
proteins
TX
TR
$OH^- +$
$OH^{\cdot}$
$TX-H_2$
NADPH
H_2O
NADP


TNF-α
Activating Caspase
Various apoptotic signals
Reactive Oxygen Species
MITOCHONDRIA
Bax
Reactive Oxygen Species
Bcl-2
AIF
Pro-caspases Cyt c
Executing caspases
Degradation phase