Chapter 3
The Mitochondrial Death Pathway

Anas Chalah and Roya Khosravi-Far*

Abstract Mitochondria have long been known to be critical for cell survival due to their role in energy metabolism. However, not until the mid-1990s did it become evident that mitochondria are also active participants in programmed cell death (PCD). This chapter focuses mainly on the role the mitochondria in mammalian cell death and cancer progression and therapy.

Keywords apoptosis, death receptors, mitochondria, bid, membranes, phospholipases, cardiolipin

1 Introduction

Apoptosis, or programmed cell death (PCD), is an evolutionarily conserved mechanism for the selective removal of aging, damaged or otherwise unwanted cells (Abe et al., 2000; Degli Esposti, 1999; Lawen, 2003; Ozoren and El-Deiry, 2003; Peter and Krammer, 1998; Strasser et al., 2000; Thorburn, 2004). It is an essential component of many normal physiological processes such as embryogenesis, normal tissue development, and the immune response (Vaux and Korsmeyer, 1999). Thus, regulation of apoptosis is critical for tissue homeostasis and its deregulation can lead to a variety of pathological conditions including carcinogenesis and chemoresistance (Burns and El-Deiry, 2003; Daniel et al., 2001; Green and Evan, 2002; Ozoren and El-Deiry, 2003; Sheikh and Huang, 2004; Thompson, 1995; Zornig et al., 2001).

Apoptosis is mediated primarily through the activation of specific proteases called caspases (cysteinyl, aspartate-specific proteases) (Algeciras-Schimnich et al., 2002;

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Ozoren and El-Deiry, 2003; Salvesen and Dixit, 1997; Stegh and Peter, 2001; Thorburn, 2004). Caspases are effectors of cell suicide and cleave multiple substrates, leading to biochemical and morphological changes that are characteristic of apoptotic cells(Abe et al., 2000; Strasser et al., 2000). These alterations include: mitochondrial outer membrane permeabilization; cell membrane remodeling and blebbing; exposure of phosphatidylserine (PS) at the external surface of the cell; cell shrinkage with cytoskeletal rearrangements; nuclear condensation; and DNA fragmentation (Ashkenazi and Dixit, 1999; Green and Evan, 2002; Lawen, 2003; Peter and Krammer, 2003; Schulze-Osthoff et al., 1998; Thorburn, 2004). These morphological changes culminate in the formation of apoptotic bodies that are normally eliminated by phagocytosis (Geske and Gerschenson, 2001; Wallach, 1997). In mammalian systems, the extrinsic death receptor pathway and the intrinsic mitochondrial pathway are the two major signaling systems that result in the activation of the executioner/effector caspases and the consequent demise of the cell (Abe et al., 2000; Ozoren and El-Deiry, 2003; Peter and Krammer, 2003; Strasser et al., 2000; Thorburn, 2004). In many cell types, including cancer cells, activation of the extrinsic pathway also engages the mitochondrial pathway for full execution of cell death (Jaattela, 2004; Khosravi-Far and Esposti, 2004; Kroemer, 2003; Newmeyer and Ferguson-Miller, 2003; Thorburn, 2004). Thus, many apoptotic signals merge at the mitochondria, and thus mitochondria have been termed “gatekeepers” of the apoptotic machinery (Jaattela, 2004; Khosravi-Far and Esposti, 2004; Kroemer, 2003; Newmeyer and Ferguson-Miller, 2003; Thorburn, 2004).

As gatekeepers, the proteins comprising the intrinsic mitochondrial pathway are the major mediators of the cytotoxic effects of many chemotherapeutic agents and radiation therapy (Brenner et al., 2003; Costantini et al., 2000; Debatin et al., 2002; Hersey and Zhang, 2003). Cancer cells often evade this apoptosis and develop chemoresistance and radioresistance. Indeed, disruption of the mitochondrial apoptotic machinery has been observed in many tumors (Daniel et al., 2001; Morisaki and Katano, 2003). It is also likely that disruption of the mitochondrial machinery or mutations in the mitochondrial DNA could play a role in cancer initiation. Because of the central role of mitochondria in these processes, various components of the mitochondrial machinery can be targets for novel therapeutic strategies.

2 The Mitochondrial Pathway of Apoptosis

Mitochondria are thought to be the primary organelles involved in mediating most apoptotic pathways in mammalian cells (Green and Kroemer, 2004; Kroemer, 2003; Newmeyer and Ferguson-Miller, 2003; Ravagnan et al., 2002; Sorice et al., 2004; Zamzami and Kroemer, 2001). Mitochondria are engaged via the intrinsic pathway of cell death, which can be initiated by a variety of stress stimuli, including ultraviolet (UV) radiation, γ-irradiation, heat, DNA damage, the actions of some oncoproteins and tumor suppressor genes (i.e., P53), viral virulence factors, and most chemotherapeutic agents (Fig. 3.1) (Kroemer, 2003). These diverse forms

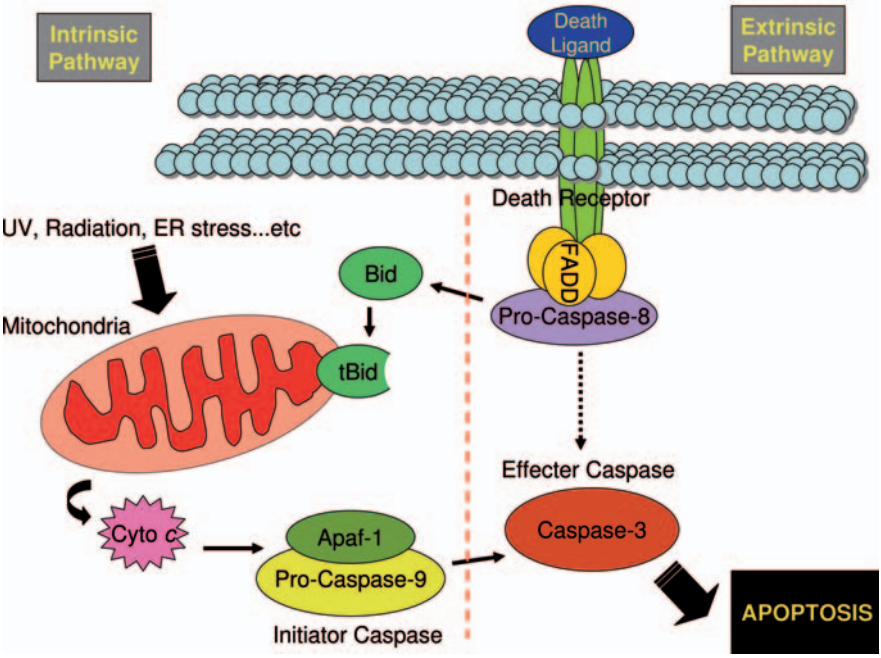

Fig. 3.1 Schematic representation of the intrinsic and extrinsic apoptotic pathways

of stress are sensed by multiple cytosolic or intraorganellar molecules. Transduction of these signals to the mitochondria ultimately results in alterations of the outer mitochondrial membrane (OM) (Esposti et al., 2003; Green and Kroemer, 2004; Kuwana et al., 2002; Newmeyer and Ferguson-Miller, 2003; Zamzami and Kroemer, 2001). These changes in the OM then lead to increased permeability to proteins that normally reside between the OM and the inner mitochondrial membrane (IM), enabling these proteins to escape the mitochondria and diffuse into the cytosol.

The mitochondrial pathway of apoptosis can also be activated in response to death ligands. In a majority of cells (type II cells), including tumor cells, extracellular death signals engage the mitochondria in a way that is equivalent to the intrinsic pathway (Abe et al., 2000; Algeciras-Schimnich et al., 2002; Ozoren and El-Deiry, 2002; Peter and Krammer, 1998). In these cells, signals originating from the death ligand-induced activation of caspase-8 and caspase-10 bifurcate into two arms, one of which directly engages mitochondria via a sequence of events causing activation of the effector caspases (i.e., caspase-3). The second arm promotes the cleavage of noncaspase substrates, such as Bid, inducing changes in the mitochondrial OM and the release of apoptogenic factors and activation of caspase-9, which then cooperates with the less-efficient activation of caspase-8 in these cells.

3 The Release of Proapoptotic Factors

Mitochondria contain and release many soluble proteins that are involved in the apoptotic cascade (Fig. 3.2) (Daniel et al., 2001; Debatin et al., 2002; Green and Kroemer, 2004; Reed, 2004). The variety of mitochondrial proteins participating in this pathway indicates the pivotal role of these organelles in determining cellular fates. Bcl-2 family members control apoptosis by regulating the permeabilization of the mitochondrial membrane (Chao and Korsmeyer, 1998; Cory et al., 2003; Daniel et al., 2001). The release of mitochondrial proteins, including cytochrome *c*, apoptosis-inducing factor (AIF), second mitochondria-derived activator of caspases (Smac/Diablo), high-temperature requirement A2 (HtrA2/Omi), and endonuclease G, is believed to play a pivotal role in inducing PCD (Martinou and Green, 2001; Zamzami and Kroemer, 2001).

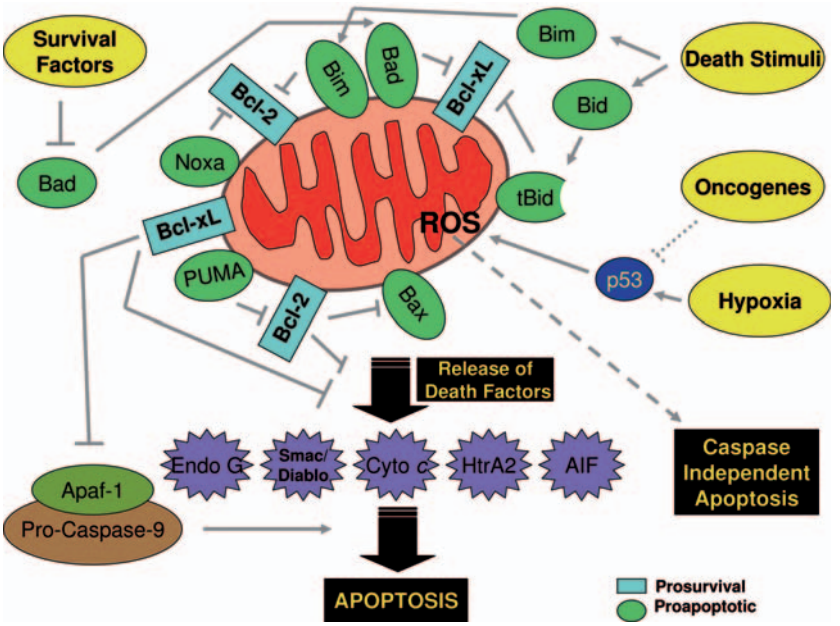

Fig. 3.2 Mitochondrial membrane permeabilization is regulated by an elegant balance of opposing actions of proapoptotic and antiapoptotic Bcl-2 family members. Bax, Bad, and Bak promote the release of cytochrome *c* and AIF through the formation of transmembrane channels across the mitochondrial outer membrane, while Bcl-2 and Bcl-X_L delay this release and abort the apoptotic response, leading to cell survival. Besides the release of mitochondrial proapoptotic components, the loss of mitochondrial membrane integrity results in the loss of many essential biochemical cellular functions such as ATP synthesis and results in the generation of reactive oxygen species (ROS). The increased levels of ROS are directly linked to the oxidation of lipids, proteins, and nucleic acids

4 Cytochrome *C*

Cytochrome *c* (Cyt *c*), a small (13 kDa) nuclear encoded mitochondrial protein, was the first protein identified as being released from mitochondria upon apoptosis. It is considered a key regulator of apoptosis because once it is released from the mitochondrial intermembrane space (IMS), the cell is irreversibly committed to death (Green and Evan, 2002; Kluck et al., 1997; Zhivotovsky et al., 1998a; Zhivotovsky et al., 1998b) and Cyt *c* is synthesized in the cytosol and translocates to the mitochondria as an unfolded apoprotein through the TOM (translocase in the OMM) complex (Diekert et al., 2001). The driving force for translocation of apo-Cyt *c* into the IMS appears to be its interaction with the enzyme cytochrome c heme lyase (Dumont et al., 1991; Mayer et al., 1995).

The release of cytochrome *c* to the cytosol is considered among the major steps in the intrinsic death pathway (Kluck et al., 1997; Newmeyer and Ferguson-Miller, 2003; Zhivotovsky et al., 1998a). Once it escapes to the cytosol, it is captured by the apoptosis protease activating factor (APAF-1), a 130 kDa adaptor protein (Soengas et al., 1999; Zou et al., 1999). Prior to binding Cyt *c*, APAF-1 is virtually inactive. Once bound to Cyt *c*, the APAF-1 monomer goes through a cytochrome *c*-induced conformational change that promotes its activation. Further oligomerization occurs, resulting in a cartwheel-shaped heptameric structure containing seven Cyt *c*/APAF-1 complexes. This larger multiprotein complex is termed the apoptosome (Acehan et al., 2002; Adrain et al., 2001; Adrain et al., 1999; Srinivasula et al., 1999). Pro-caspase-9 is recruited to the apoptosome through its CARD domain, promoting its cleavage and converting it to an active protease (Adrain et al., 1999). Consequently, caspase-9 dissociates from the complex and goes on to activate effector caspases (3, 6, and 7) which collectively orchestrate the execution of apoptosis (Slee et al., 1999; Srinivasula et al., 1999; Zou et al., 1999).

5 Apoptosis-Inducing Factor

The precursor of the protein AIF is synthesized in the cytosol and imported into mitochondria (Susin et al., 1999). It contains an N-terminal mitochondrial localization sequence (MLS) which is cleaved upon its mitochondrial translocation to form the mature 57 kDa AIF (Susin et al., 1999). Under apoptosis-inducing conditions, AIF translocates through the permeabilized mitochondrial outer membrane to the cytosol (Cande et al., 2002; Susin et al., 1999). Subsequently, AIF is transported to the nucleus where it induces ATP-independent nuclear chromatin condensation, as well as large-scale DNA fragmentation (Cande et al., 2002; Susin et al., 1999). In contrast to cytochrome *c*, AIF acts in a caspase-independent fashion and does not require the presence of cytosolic factors to induce apoptotic features in the nuclei (Lorenzo et al., 1999; Miramar et al., 2001; Susin et al., 1999; Zamzami and Kroemer, 2001). Moreover, AIF translocation occurs in Apaf-1-null mice which

fail to activate the executioner caspase (Cecconi et al., 1998). However, some studies indicate that crosstalk does occur between AIF and the apoptotic caspase cascade (Cande et al., 2002). For instance, AIF was observed to trigger the release of cytochrome *c* from isolated mitochondria (Susin et al., 1999). Additionally, AIF interacts with heat-shock protein 70 (Hsp70), a known protective factor and inhibitor of Apaf-1-dependent caspase activation (Ravagnan et al., 2002).

6 Smac/Diablo

Second mitochondria-derived activator of caspases (Smac) is a 22 kDa mitochondrial protein also known as direct IAP-associated binding protein with low pI (Diablo). Inhibitors of apoptosis (IAP) family members have the ability to interact and inhibit the enzymatic activity of caspases through their baculovirus inhibitor repeat (BIR) functional motif (Deveraux and Reed, 1999; Miller, 1999). Smac/Diablo was first identified as a mammalian IAP (Srinivasula et al., 1999; Verhagen and Vaux, 2002). Specifically, XIAP, c-IAP1, and c-IAP2 are proapoptotic factors regulated by Smac/Diablo (Ekert et al., 2001; Srinivasula et al., 1999; Verhagen and Vaux, 2002). The Smac/Diablo precursor is synthesized in the cytosol, then imported to the mitochondria where it is cleaved and activated. A mature form of Smac/Diablo is released to the cytosol under apoptotic conditions. Unlike cytochrome *c*, which directly activates APAF-1 and caspase-9, Smac/Diablo binds to the BIR domains of multiple IAP members, antagonizing them and promoting indirect caspase activation (Ekert et al., 2001; Srinivasula et al., 1999; Verhagen and Vaux, 2002). Smac/Diablo and cytochrome *c* were found to be released from the mitochondria at around the same time. Moreover, the release was found to coincide with mitochondrial membrane potential depolarization (Rehm et al., 2003; Springs et al., 2002; Verhagen and Vaux, 2002). However, a recent study presented evidence suggesting that the release of Smac/Diablo may, in fact, depend on the release of cytochrome *c* (Hansen et al., 2006).

7 HtrA2/Omi

HtrA2, also referred to as Omi, is a mitochondrial protein that belongs to the family of serine proteases. This proapoptotic protein is expressed as a 50 kDa precursor that is cleaved at the N-terminal, upon translocation to the mitochondria, to generate the active 36 kDa protein (Hegde et al., 2002; Martins et al., 2002; Suzuki et al., 2001; Verhagen and Vaux, 2002). Similar to cytochrome *c* and Smac/Diablo, mature HtrA2/Omi localizes to the IMS (Hegde et al., 2002; Suzuki et al., 2004). Its release to the cytosol is stimulated by apoptotic triggers. Upon its release, HtrA2/Omi binds directly to the BIR domain of IAPs and inhibits their caspase-inhibitory activity (Suzuki et al., 2001). The first four N-terminal amino acids of the mature HtrA2 protein (AVPS) constitute the IAP-binding motif.

In addition to the proapoptotic effect of IAP binding and inhibition, Omi/HtrA2 appears to utilize its serine protease activity to induce an IAP inhibition-independent, caspase-independent apoptosis (Hegde et al., 2002; Suzuki et al., 2001). Recently, it was reported that the proapoptotic serine protease activity of HtrA2/Omi also plays a significant role in antagonizing IAPs. The observed HtrA2 cleavage of c-IAP produced significant caspase activation and sensitized cells to apoptosis (Yang et al., 2006).

8 Endonuclease G

As with most mitochondrial proteins, Endonuclease G is expressed as a precursor in the cytosol. Upon its translocation to the mitochondria, the 33 kDa protein is cleaved to a 28 kDa mature form (Cote and Ruiz-Carrillo, 1993). During apoptosis, endonuclease G is released from the mitochondrial IMS and translocates to the nucleus, where it causes oligonucleosomal DNA fragmentation (Li et al., 2001; van Loo et al., 2001). Endonuclease G release appears to be dependent on caspase activation downstream of mitochondria (Arnoult et al., 2003). Interestingly, endonuclease G-induced DNA degradation was observed to be caspase-independent (Li et al., 2001; Susin et al., 1999), suggesting an important role for endonuclease G in bringing about caspase-independent cell death.

9 Mitochondrial Proteins and Caspase Activation

Among the various proteins that leak out of mitochondria, a few, such as cytochrome *c*, play a major role in promoting caspase activation. (Kluck et al., 1999; Saelens et al., 2004) These apoptogenic factors are released in a hierarchical manner during cell death. Upon activation of the intrinsic pathway, cytochrome *c*, Htr2A/Omi and Smac/Diablo are released first, with similar kinetics (Saelens et al., 2004). The subsequent release of AIF and endonuclease G (Arnoult et al., 2003; Penninger and Kroemer, 2003) is associated with more severe damage to both the outer and inner membranes. Notably, cytochrome *c* has been shown to be directly involved in the mediation of cell death, as it is indispensable for the activation of Apaf-1 and subsequent formation of the apoptosome (Arnoult et al., 2003).

The apoptosome itself is a platform for recruiting and facilitating the autocatalytic activation of pro-caspase-9, the apical caspase of the intrinsic pathway of apoptosis (Adams and Cory, 2002; Baliga and Kumar, 2003; Cain et al., 2002; Chinnaiyan, 1999; Hill et al., 2003; Salvesen and Renatus, 2002; Shi, 2002). The activation of caspase-9 leads to the local accumulation of zymogens, promoting an autocatalytic process of downstream caspase activation (Adams and Cory, 2002; Baliga and Kumar, 2003; Cain et al., 2002; Chinnaiyan, 1999; Hill et al., 2003; Salvesen and Renatus, 2002; Shi, 2002). However, the apoptosome requires additional

regulatory factors, including Smac/Diablo, for full activation of the caspase cascade. Smac/Diablo interacts with several IAPs to release them from their inhibitory interaction with pro-caspase-9 and other caspases (Adams and Cory, 2002; Baliga and Kumar, 2003; Cain et al., 2002; Shi, 2002). Smac/Diablo is also present in the mitochondria, where it is directly attached to the OM and is released upon alterations in the OM permeability (Cain et al., 2002; Saelens et al., 2004).

10 Mechanisms of Mitochondrial Protein Release

The exact mechanism by which mitochondrial proapoptotic components are released from the IMS is a matter of a long and ongoing debate. Currently, two general mechanisms are considered: nonspecific and specific release (Lim et al., 2001). The opening of the permeability transition pore (PTP) located in the mitochondrial IMS is proposed as the first possible mechanism. The permeability pore is comprised of three proteins: cyclophilin D, adenine nucleotide translocator (ANT), and voltage-dependent anion channel (VDAC), a matrix, an inner membrane, and an outer membrane protein, respectively (Crompton, 1999). The opening of the PTP triggers many processes, including (A) loss of the proton gradient produced by the electron transport machinery; (B) leakage of cellular water into the mitochondrial matrix, resulting in the gradual swelling of the IMS and the rupturing of the inflexible OM (Green and Kroemer, 2004); and (C) leakage of apoptotic factors from the IMS into the cytoplasm, which begins the cascade of proteolytic activities leading ultimately to nuclear damage and cell death (Brenner et al., 2000; Dejean et al., 2006; Kroemer, 2003; Marzo et al., 1998a; Marzo et al., 1998b). This mechanism represents a nonspecific release mode for proapoptotic mitochondrial mediators. However, the physical outer membrane disruption theory fails to explain the release of proapoptotic factors such as cytochrome *c* and AIF in the absence of any loss of outer membrane structural integrity (Dejean et al., 2006).

The second suggested mode of release involves the opening of large outer membrane channels that would allow cytochrome *c* and other IMS proteins to move into the cytosol. In contrast with the other scenarios, this model would leave the outer membrane largely intact. A benefit of this model is that there is no need for the mitochondrial matrix to swell. This better fits with the evidence that mitochondrial morphology remains the same in most cell death in vivo. Several outer membrane channels, including the VDAC and mitochondrial apoptosis-induced channel (MAC), have been targeted as possible specific regulators of mitochondrial release. Both provide aqueous pathways through the hydrophobic environment of the mitochondrial membrane.

VDAC is a 30 kDa highly conserved voltage-dependent, ion-selective, mitochondrial OM protein. The OM is densely packed with VDAC proteins which form barrel structures that enclose 3 nm internal diameter channels. VDAC can switch between two functional states, open and partially open. The “open” state is defined by large conductance and anion selectivity, while the “partially open” state is

defined by lower conductance (about half that of the fully open state) and cation selectivity. The voltage-dependent change between these two states is widely attributed to structural rearrangements that lead to changes of size and charge distributions within the channel (Colombini et al., 1996; Mangan and Colombini, 1987; Thomas et al., 1993).

MAC was first identified in 2001. It is a mitochondrial outer membrane channel that, according to some reports, forms at early stages of the intrinsic apoptotic pathway (Dejean et al., 2006; Guo et al., 2004). Alternatively, other studies have reported the formation of MAC at late stages of the extrinsic apoptotic pathway (Guihard et al., 2004). MAC was found to be slightly cation-selective, and unlike VDAC, voltage-independent (Dejean et al., 2005; Guo et al., 2004). MAC activity was found to be induced by apoptosis and regulated by Bax, a proapoptotic Bcl-2 family protein. Bax translocation to the mitochondria was linked to MAC formation and cytochrome *c* release (Antonsson et al., 1997; Dejean et al., 2006; Guo et al., 2004; Saito et al., 2000; Schendel et al., 1997). Bax oligomerization is proposed to form MAC channels (Cheng et al., 2001; Dejean et al., 2006; Wei et al., 2001). The pore diameter of the MAC channel was measured to be ~4 nm, which is proposed to allow for the release of the ~3 nm diameter cytochrome *c* (Pavlov et al., 2001).

11 The Bcl-2 Family of Proteins and Regulation of the Mitochondrial Pathway to Cell Death

The process of mitochondrial release of proapoptotic factors such as cytochrome *c* is elegantly regulated through members of the Bcl-2 family (Fig. 3.2) (Antonsson et al., 1997; Cory et al., 2003; Danial and Korsmeyer, 2004; Green and Kroemer, 2004; Schendel et al., 1997). In mammals, the antiapoptotic members of this family include Bcl-2, Bcl-X_L, and Bcl-W, while the proapoptotic members include Bax, Bak, Bad, Bik, Bim, and Bid. The proapoptotic family members are further classified based on domain sequence homology into two groups: one that contains multiple BH domains and one that contains only the BH3 domain (Cheng et al., 2001; Fiers et al., 1999; Kuwana and Newmeyer, 2003; Wei et al., 2001). The fate of the cell depends to a great degree on the precious balance of function between these proapoptotic and antiapoptotic Bcl-2 proteins. Studies have shown that Bax, Bad, and Bak promote the release of AIF and cytochrome *c*, while Bcl-2 and Bcl-X_L delay the release and abort the apoptotic response, promoting cell survival (Cory and Adams, 2002; Yang et al., 1997).

It is believed that Bcl-2 family members regulate the apoptotic response by controlling mitochondrial membrane permeabilization (MMP) (Green and Kroemer, 2004). The proapoptotic proteins Bax and Bak have been shown to contribute to the formation of transmembrane channels across the mitochondrial OM, leading to the escape of AIFs (Dejean et al., 2005; Korsmeyer et al., 2000; Kuwana et al., 2002; Nechushtan et al., 2001; Wei et al., 2001). Bcl-2, Bcl-W, and Bcl-X_L are, on the other hand, believed to prevent pore formation and to inhibit the release

of cytochrome *c* from the mitochondria (Kluck et al., 1997; Yang et al., 1997). Moreover, heterodimerization of Bax or Bad with Bcl-2 or Bcl-X_L is thought to inhibit their protective effect.

Bid is a potent proapoptotic protein that is normally located in the cytosol, but also shuttles through the surfaces of intracellular membranes due to its lipid-interacting capacity. Bid plays an important role in the mitochondrial pathway to apoptosis as it has been identified as the link between the death receptor signal and the release of cytochrome *c*. Activated caspase-8 engages the intrinsic apoptotic pathway through the truncation of Bid (Li et al., 1998; Luo et al., 1998). Upon death signaling, activated caspase-8 cleaves Bid (26 kDa) into two fragments: a C-terminus fragment (15 kDa) and an N-terminus fragment (11 kDa) (Luo et al., 1998). The 15 kDa fragment, which contains the BH3 domain, is termed truncated Bid or tBid. This functional fragment translocates to the mitochondria where it interacts with several proteins through its BH3 domain (Wang et al., 1996). There are two modes of Bid proapoptotic action. (1) In the BH3-dependent mode, Bid interacts with the antiapoptotic Bcl-X_L through its BH3 domain and prevents the formation of the Bcl-X_L/Apaf1 antiapoptotic complex. (2) In the BH3-independent mode, after truncation, Bid is proposed to form selective channels similar to BAX through its structural motifs (Chou et al., 1999; McDonnell et al., 1999). Moreover, tBid has been shown to induce the oligomerization of Bax and Bak, resulting in MAC formation and the subsequent release of proapoptotic cytochrome *c* (Eskes et al., 2000; Wei et al., 2000).

The mitochondrial receptor for caspase-cleaved Bid is thought to be cardiolipin (CL), a mitochondrial lipid (Esposti et al., 2003; Kuwana et al., 2002; Newmeyer and Ferguson-Miller, 2003; Sorice et al., 2004). CL is a glycerophospholipid that is synthesized and localized in the inner membrane of the mitochondria, making it one of its major constituents (Khosravi-Far and Esposti, 2004; McMillin and Dowhan, 2002; Schlame et al., 2000; Wright et al., 2004). This dimeric molecule apparently plays a significant role in controlling the mitochondrial membrane structure and function. Abnormal mitochondrial morphology and function have been observed in cells defective in the CL synthesis mechanism (Ohtsuka et al., 1993). It has been proposed that upon apoptotic stimulation, CL contributes to the apoptotic signal through the recruitment of cytosolic proteins such as tBid to the mitochondrial membrane. Additionally, it is thought that CL is involved in altering MMP, leading to the subsequent release of proapoptotic factors (Lutter et al., 2000).

12 Mitochondria and Oxidative Stress

Mitochondria are the sites of aerobic respiration. Energy is generated in mitochondria through the process of ATP synthesis via the oxidative phosphorylation pathway. This process, however, also results in the formation of single unpaired electrons, leading to reactive oxygen species (ROS). ROS such as hydrogen peroxide (H_2O_2), the superoxide anion (O_2^-), and hydroxyl radicals (OH) are highly

reactive molecules generated and eliminated in a balanced process in normal cells. In particular, free radicals (superoxides) are byproducts of ATP generation by the mitochondrial respiratory chain (Andreyev et al., 2005; Beyer, 1992; Raha and Robinson, 2000). Cellular energy is usually liberated from ATP molecules through the removal of single phosphate-oxygen groups, producing adenosine diphosphate (ADP). ADP is recycled in the mitochondria where it is recharged through oxidative processes to reproduce ATP. Since ROS are harmful, the balance between energy supply and energy demand is extremely critical. Any shift in this balance would introduce excess ROS to cells and would result in oxidative stress.

The damaging effect of elevated levels of ROS is thought to be due to the highly reactive free electrons available to form stable chemical bonds. While H_2O_2 is free to escape the mitochondrion, both the superoxide anion and hydroxyl radicals have limited diffusion, and are more likely to contribute to inner membrane damage of mitochondria (Szeto, 2006). Several studies have demonstrated a direct relationship between mitochondrial ROS and the mitochondrial apoptotic pathway. For example, the release of cytochrome *c* to the cytosol has been linked to mitochondrial oxidation (Shidoji et al., 1999). It is believed that the release mechanism might involve the opening of mitochondrial PTPs (Vieira et al., 2001). Several antioxidant compounds, such as ascorbic acid (vitamin C), α-tocopherol (vitamin E), and ubiquinol are naturally present in the cell and act to protect against the effects of ROS (Sies and de Groot, 1992).

13 Mitochondria and Cancer

Given the important roles mitochondria play in cellular energy metabolism, free radical formation and PCD, defects in mitochondrial function are suspected to contribute to the development and progression of cancer and to resistance to therapy (Bettaieb et al., 2003; Brenner et al., 2003; Costantini et al., 2000; Debatin et al., 2002; Hersey and Zhang, 2003; Jaattela, 2004; Kasibhatla and Tseng, 2003; Kim et al., 2004). Defective apoptosis is one of the hallmarks of tumorigenicity and is implicated in multiple stages of tumor progression (Burns and El-Deiry, 2003; Hanahan and Weinberg, 2000; Ozoren and El-Deiry, 2003). Furthermore, the ability of tumor cells to escape apoptosis plays a key role in promoting resistance to conventional chemotherapy and radiation therapy (Abe et al., 2000; Barnhart et al., 2004; Daniel et al., 2001; El-Deiry, 1997; Thompson, 1995; Zornig et al., 2001).

A link between mitochondria and cancer progression was suggested over half a century ago when Warburg reported the role of mitochondria in cellular energy metabolism. This phenomenon was coined the "Warburg effect." The Warburg effect suggested that the development of an injury to the respiratory machinery is an important event in carcinogenesis (Warburg, 1951). This injury results in compensatory increases in glycolytic ATP production to fulfill the energy needs of tumor cells. Since then, preferential reliance on glycolysis over the oxidative metabolism has been shown to correlate with tumor progression in several types of

cancer (Semenza et al., 2001). Since the initial report of the Warburg effect, a number of cancer-related mitochondrial defects have also been identified (Brenner et al., 2003; Carew and Huang, 2002; Debatin et al., 2002; Jaattela, 2004). These defects include altered expression and activity of respiratory chain subunits and glycolytic enzymes, changes in oxidation of NADH-linked substrates and mutations in mitochondrial DNA. Thus, the differences in energy metabolism between normal cells and cancer cells constitute a biochemical basis for the development of therapeutic strategies that might selectively kill cancer cells in their compromised respiratory state.

Furthermore, dysregulation of members of the Bcl-2 family has been detected in a variety of malignancies, especially hematological cancers. Bcl-2 itself was originally discovered as an oncogene in B cell lymphoma Danial and Korsmeyer, 2004. Additionally, overexpression of Bcl-2 has been detected in AML and non-Hodgkin's lymphomas. Dysregulation of other Bcl-2 family proteins have also been detected in other cancers; for example, increased expression of Mcl-1 has been detected in relapsed AML and multiple myeloma. Increased expression levels and mutations in the promoter of the *mcl-1* gene have also been observed in chronic lymphoblastic leukemias. These studies reiterate that changes to the mitochondrial-associated proteins, mainly members of the Bcl-2 family, are directly involved in tumor progression.

Additionally, there is some evidence that alterations in the mitochondrial DNA could also be involved in cancer progression. Besides hosting hundreds of nuclear encoded proteins, mitochondria have their own DNA that encodes 13 mitochondrial proteins (Schatz, 1995; Singh et al., 1999). Mutations in mtDNA could occur during oxidative phosphorylation involving ROS. Investigations of human bladder, lung, neck, and head primary tumors revealed a high percentage of mtDNA mutation (~50%) in these tumors (Fliss et al., 2000). These observations suggest a link between cancer development and mitochondrial dysfunction; however, they do not present a clear answer to whether mitochondrial DNA mutation is simply a result, or rather the cause, of alterations in PCD.

Mitochondria also play an important role in resistance to chemotherapy and radiation therapy. Since mitochondria are integrators of apoptotic signaling pathways, induction of apoptosis in many cell types leads to the induction of MMP (Brenner et al., 2003; Kroemer, 2003). MMP defines the point of no return in most PCD pathways and is regulated by pre-mitochondrial signal transduction pathways. These pathways involve caspase-dependent and caspase-independent mechanisms, members of the Bcl-2 family of proteins and changes in the composition of mitochondrial membranes (Bettaieb et al., 2003; Brenner et al., 2003; Green and Kroemer, 2004; Kim et al., 2004; Kroemer, 2003; Kuwana et al., 1998; Newmeyer and Ferguson-Miller, 2003; Peter and Krammer, 1998; Ravagnan et al., 2002; Sorice et al., 2004; Waterhouse et al., 2001; Zamzami and Kroemer, 2001). In response to MMP, proapoptotic factors are released into the cytosol to trigger the execution of cell death. This is likely due to the opening of protein channels such as the VDAC. Under pathological conditions, cancer cells escape from apoptosis and/or become resistant to treatment by affecting MMP (Bettaieb et al., 2003; Debatin et al., 2002; Hersey and Zhang, 2003; Kim et al.,

2004). Therefore, overcoming abnormalities in tumor cells that suppress MMP could lead to therapeutic targets by generating a potent proapoptotic stimulus. Additionally, since MMP is an early event in apoptosis, strategies to detect this process can be useful in assessing the response to chemotherapy.

Mutations in mtDNA have been implicated in the cellular response to chemotherapy. For example, Singh et al. (1999) examined the response of a tumor cell line lacking mitochondrial DNA to several anticancer drugs, including adriamycin (a DNA-interacting drug widely used in chemotherapy for its role in binding DNA and stopping the process of replication). Cancer cells lacking mtDNA showed great chemotherapy resistance, indicating an important role of the mitochondrial genome in regulating the cellular response to therapeutic agents. Similar findings were also reported in A549 non-small-cell lung cancer cell lines and their rho0 derivatives in which mitochondrial DNA has been eradicated (Lo et al., 2005). The parental cell line showed increased sensitivity to chemotherapy when compared with the mtDNA-compromised derivative cell line. Notably, the restoration of mtDNA restored chemosensitivity of the resistant cell line (Lo et al., 2005).

14 Targeting Mitochondria in Cancer Therapy

As mitochondria are gatekeepers of apoptotic signals, targeting mitochondria to induce apoptosis of malignant cells is an important therapeutic strategy. In the past several years, extensive research has focused on screening for chemical compounds, small molecules and peptides that could target the mitochondria. Therapeutic tactics have included strategies that involve the Bcl-2 family proteins, activation of PTPs, the respiratory chain, mitochondrial DNA depletion, and selective targeting of ROS-stressed malignant cells, as well as targeting inhibitors of apoptosis such as IAPs (Dias and Bailly, 2005). Targeting the antiapoptotic members of the Bcl-2 family, namely Bcl-2 and Bcl-X_L, and targeting the PTP are among the most studied mechanisms (Dias and Bailly, 2005; O'Neill et al., 2004; Shangary and Johnson, 2003; Walensky, 2006). Targeting of the Bcl-2 family of proteins is discussed in Chapter 8. Here, we will briefly describe strategies for targeting and activation of the PTP.

15 Targeting and Activation of the Permeability Transition Pore

The induction of proapoptotic protein release through increased PTP formation and opening has been explored in the recent years as a possible mechanism for cancer treatment. As a chemotherapeutic approach, this method involves perturbation of the mitochondrial membrane through direct targeting of the components of the

membrane permeability transition pore complex (PTPC) (Brenner et al., 2003; Costantini et al., 2000; Debatin et al., 2002; Fantin and Leder, 2006; Galluzzi et al., 2006; Khosravi-Far and Esposti, 2004; Morisaki and Katano, 2003; Reed, 2004). Additionally, alterations in energy metabolism, such as depletions in ADP and ATP, can also facilitate formation of the PTPC.

In addition to therapeutic strategies that target Bcl-2 family members, several chemotherapeutic agents such as paclitaxel or etopiside have been shown to induce opening of the PTPC, albeit at high concentrations. Additionally, several experimental anticancer agents act directly on the components of the PTPC. For example, the synthetic retinoid CD437, arsenic acid and lonidamine are inhibitors of ANT (Debatin et al., 2002; Fantin and Leder, 2006; Galluzzi et al., 2006). Arsenic acid also inhibits the VDAC. Hexokinase, which is a component of the PTPC and a major player in maintaining the malignant state of transformed cells, is also inhibited by lonidamine (Debatin et al., 2002; Fantin and Leder, 2006; Galluzzi et al., 2006). Additionally, jasmonates are known to act selectively and directly on cancer cell mitochondria in a PTPC-mediated mechanism, resulting in membrane depolarization, swelling, and the release of cytochrome *c* (Rotem et al., 2005) leading to apoptosis of tumor cells. Similarly, lamellarins are another group of anticancer drugs that target mitochondria of cancer cells and induce permeability transition effects (Kluza et al., 2006).

16 Conclusions and Future Prospects

Mitochondria are the power generators of the cell due to their involvement in glucose metabolism, and they are "gatekeepers" of the cell involved in integrating apoptotic signals in majority of cells. Because tumor cells rely on glycolysis and since evasion of apoptosis is one of the hallmarks of cancer, mitochondria therefore play a central role in cancer cell biology. The intrinsic and extrinsic death pathways leading to changes in mitochondrial permeability; the components of the PTPC, including members of the Bcl-2 family; apoptogenic factors and their regulators, and mutations in mtDNA have been studied extensively in the past for their contributions to cancer progression or resistance to therapy. These constitute an extensive list of targets that could induce apoptosis, some with possible specificity for cancer cells. Therapeutic agents against many of these targets, including Bcl-2 family members and components of the PTP, are currently at various stages in the development pipeline. The ultimate goal of these studies is to generate novel mitotoxic agents that can selectively induce apoptosis of cancer cells and reduce the possibility of resistance.

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