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The mechanisms of apoptosis in biology and medicine: a new focus for ophthalmology

A. TEMPESTINI, N. SCHIAVONE*, L. PAPUCCI*, E. WITORT, A. LAPUCCI, M. CUTRÌ, M. DONNINI, S. CAPACCIOLI*

Research Group on Apoptosis applied to Ophthalmology (Firenze Division), Department of Experimental Pathology and Oncology-University of Firenze - Italy

ABSTRACT. *Defects in apoptosis (programmed cell death) have recently emerged as being closely involved in the pathogenesis of most ocular diseases and, therefore, apoptosis is now a topic of exponential interest in ophthalmology. This review summarizes recent works on mechanisms of apoptosis, from its initiation and modulation to the switching-on of its execution machinery. Interactions of cell death with cell division programs to orchestrate ontogenesis, aging, and adult life and their alterations in human diseases are pointed out. Two main apoptotic signaling pathways are identified: a death receptor-dependent (extrinsic) pathway and a mitochondrion-dependent (intrinsic) pathway. Mitochondrion harbors both anti-apoptotic (Bcl-2, Bcl-X_L) and apoptotic factors (Smac/Diablo, Apaf-1, cytochrome c). Its permeability transition pore (mPTP) is the main trigger of cell suicide. The process of mPTP opening, in association with extrusion to cytoplasm of a variety of apoptotic factors, is shown. Cytochrome c is one of these apoptotic factors. When expelled to cytoplasm, this double-faced respiratory chain component assembles with two other modules, Apaf-1 and procaspase 9, to form a protein complex – the apoptosome – that starts apoptosis execution. Another respiratory chain component, the CoQ_{10}, is believed to counteract mPTP opening. What makes apoptosis particularly exciting for medicine is that its dysfunctions play a central role in the pathogenesis of several human diseases. For instance, excesses of apoptosis lead to cell loss that accompanies neurodegenerative diseases, whereas genetically determined defects of apoptosis lead to the deregulated cell proliferation typical of cancer. A variety of ophthalmologic diseases, such as post-keratectomy haze, corneal lesions, cataract, glaucoma, senile maculopathies, and genetic ocular pathologies, that underlie apoptosis dysfunctions are treated in detail in the other reviews of this issue.* Eur J Ophthalmol 2003; 13 (Suppl. 3): S11-S18

KEY WORDS. *Apoptosis, Ocular pathology, Post-keratectomy haze, Gene expression, Permeability transition pore CoQ_{10}*

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What is apoptosis?

Apoptosis is a cellular program operating in parallel with cell division by which a cell commits suicide (programmed cell death). Apoptosis is vital for all metazoan, occurs in a variety of physiologic conditions, and accompanies the entire life of organisms, including humans. From embryonic differentiation (1) to aging (2-4), apoptosis was, until recently, an undervalued life protagonist, spanning all adult life (5). Biological life requires a very tight equilibrium between cell entry and exit through tissue compartments (6). This implies the existence of cross-talking control factors that finely tune both processes. The scenario is a network

**These authors have contributed equally to this review*

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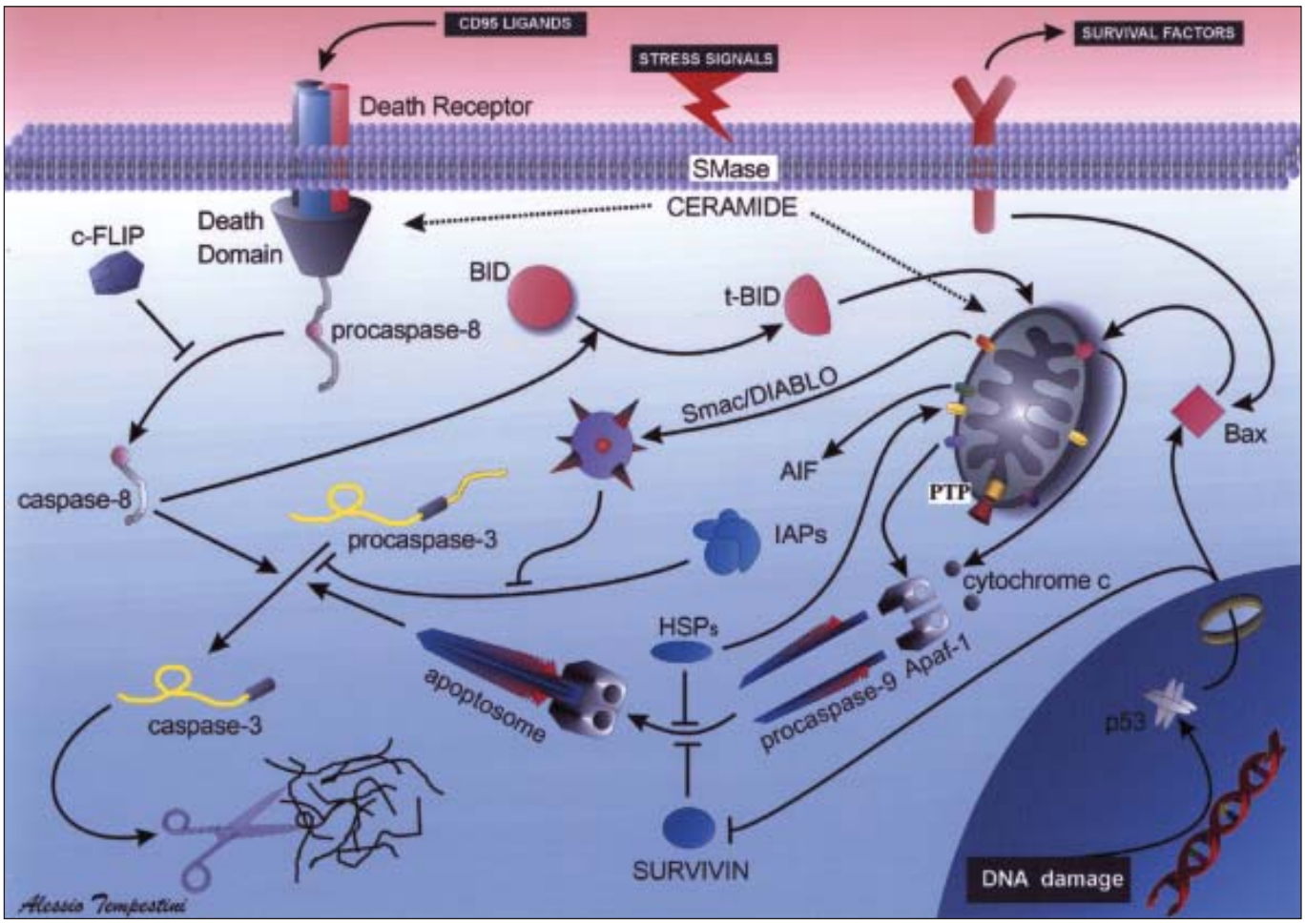

Fig. 1 - *A simplified scheme of the apoptotic machinery. The scheme is based on identification of two main death pathways: death receptor-dependent pathway and mitochondrion-dependent pathway. Death ligand binding to death receptors leads to activation of the procaspase 8. Apoptotic signal-induced opening of mitochondrion permeability transition pore recruits several cytoplasmic apoptotic members of Bcl-2 family and other proteins to form transmembrane mitochondrial channels by which several apoptosis executors (Smac/Diablo, AIF, cytochrome c, Apaf-1, and procaspase 9) are extruded to cytoplasm: hetero-oligomerization of cytochrome c, Apaf-1, and procaspase 9 within apoptosome leads to activation of procaspase 9. In turn, by means of a cascade process, either activated caspase 8 or 9 activates downstream caspases, which are the death tools of apoptotic machinery. Triggering of apoptotic machinery is counterbalanced by several antiapoptotic factors, which act on all apoptotic pathway levels, from membrane (c-FLIP) to mitochondrion (Bcl-2, Bcl-X_L), to cytoplasm (Survivin, IAP, and HSP). Although relatively complex, this scheme must be considered a virtual reality of a much more complex scenario: imagine that clicking on each point of this scheme allows us to travel through a hyper-textual world of many cross-talking molecules, through the life/death pathway cross-roads. Now we click onto mitochondrion to pass hyper-textually to Figure 2.*

of molecules, belonging to all cellular compartments, from nucleus to mitochondria, from Golgi apparatus to cell membrane (7), which are integrated into the two multiphasic programs of cell division and cell death, both constitutively expressed in the cell.

Cell death by apoptosis is also, paradoxically, a defense mechanism of organisms against cellular injuries (5). What happens when one or a few cells have been damaged? There are two alternative choices: either to repair the injured cell or to commit it to suicide. The earliest cellular response to a slight injury is the heat-shock response (8, 9): a number of evolutionarily conserved genes are activated, whose protein products (heat-shock proteins [HSP]) can repair damaged molecules. However, when injury is moderate to severe the best and most economical solution is to eliminate the damaged cell by apoptosis and to rebuild a new one by cell division. Obviously, heat-shock response

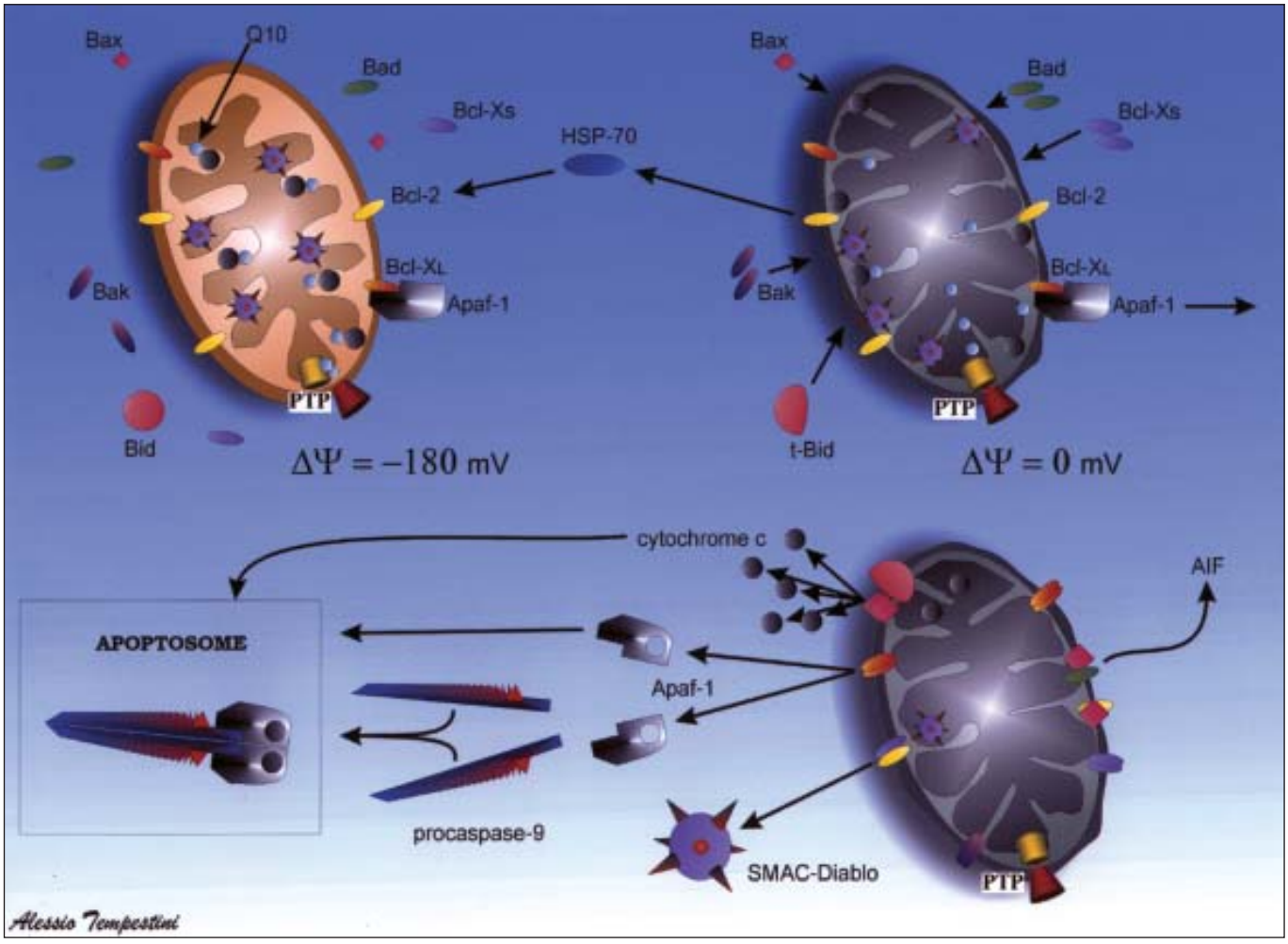

Fig. 2 - *Mitochondrion: the main switch of apoptosis execution. Its transformation from cell factory of energetic molecules to that of cell-killer molecules is shown here. Top left: In normal conditions, cytochrome c and ubiquinone Q10 work within the respiratory chain and the latter as possible mPTP opening inhibitor. Bcl-2 and Bcl-X_L act as antiapoptotic docking proteins, Apaf-1 is anchored to Bcl-X_L, and proapoptotic members of Bcl-2 family and procaspases float, inactive, in the cytoplasm. Top right: When the cellular balance between antiapoptotic and proapoptotic signals shifts in favor of cell death, a collapse of mitochondrial $\Delta\psi$ consequent to mPTP opening occurs that is associated with disruption of mitochondrion architecture and respiratory function, switching off Bcl-2 and Bcl-X_L gene expression, detachment of Apaf-1 from Bcl-X_L protein, and recruitment of apoptotic members of Bcl-2 family and t-Bid to external mitochondrial membrane. Bottom left: Mitochondrial channel formation by apoptotic member of Bcl-2 family leads to extrusion from mitochondrion to cytoplasm of a variety of apoptogenic molecules including AIF, Smac/Diablo, as well as cytochrome c, Apaf-1, and procaspase 9. Hetero-oligomerization of cytochrome c, Apaf-1, and procaspase 9 leads to formation of apoptosome and activation of caspase cascade, which leads to cell suicide. Opening of mPTP is the master switch of these events: now we click on mPTP to pass hyper-textually to Figure 3.*

and apoptosis are antithetic: for this reason HSP act as antiapoptotic factors through direct physical interaction with key components of the apoptotic machinery (10).

The apoptotic machinery

The machinery of cell suicide is maintained at a critical threshold between off/on by positive and negative regulative factors. Indeed, it is a rule for most biological processes that each apoptotic factor is often finely balanced by a relevant antiapoptotic factor. A further complicating element is that most apoptotic factors have a double identity, being endowed with quite different functions in different contexts. The dynamic balance of these positive, negative, and often double-faced factors determines

whether a cell commits suicide. Classically, two distinct classes of apoptotic signaling pathways have been identified: an extrinsic death pathway, mediated by cell membrane, and an intrinsic death pathway, whose central control point is mitochondrion (11-13). However, because mitochondrion is also the downstream target for the extrinsic pathway, we propose a scheme that distinguishes between death receptor-dependent pathway and mitochondrion-dependent pathway (Fig. 1). Both pathways lead to activation of the apoptosis-execution machinery, whose death tools are the caspases (14, 15). Caspases constitute a family of at least 14 apoptosis-specific endoproteases (from caspase 1 to caspase 14), which are constitutively expressed as inactive precursors (procaspases). When activated by means of a cascade process similar to that of blood clotting, caspases actuate apoptotic cell death, which results in cleavage of cellular substrates, from proteins to DNA (16).

The master switch of the death receptor-dependent pathway is binding of death ligands, such as tumor necrosis factor (TNF)-α and APO-1-L/Fas-L, to specific death receptors (DR), such as TNF-R and APO-1/Fas. This induces DR trimerization, engagement of adaptor molecules to cytoplasmic domain, and consequent proteolytic activation of procaspase 8: this is followed by activation of procaspase 3, which leads to apoptotic death execution (17). Membrane stresses also lead to production of a lipidic messenger of death, the ceramide, which seems to act both by the DR and by the mitochondrion-dependent pathways (18). Mitochondria are at the crossroads of life and death signaling pathways and can also collect apoptotic signals derived from membrane (survival factors withdrawal, tBid, ceramide) and nucleus (DNA damage-activated p53). This explains the "mitochondriocentric" point of view of apoptosis emerging from the recent literature (19). The trigger of caspase cascade in the mitochondrion-dependent apoptotic pathway is procaspase 9. As shown in Figure 2, when the balance of mitochondrial antiapoptotic (Bcl-2, Bcl-X_L, Mcl-1, Bfl-1/A1, Bcl-W, Bcl-G) or proapoptotic (Bax, Bak, Bok, Bad, Bid, Bcl-X_S, Bik, Bim, Krk, Mtd, Nip3, Nix, Noxa, Bcl-B) members of the Bcl-2 family shifts in favor of cell death, the mitochondrion-dependent death pathway is switched on by opening of a high-conductance nonselective mitochondrial channel (20) – namely, the mPTP – and extrusion to cytoplasm of a variety of apoptogenic molecules, such as cytochrome c, Apaf-1, procaspase 9, apoptosis inducing factors (AIF), and Smac/Diablo. Cytochrome c, Apaf-1, and procaspase 9 form a hexameric death tool known as apoptosome that activates the caspase cascade starting from caspase 3. The first mitochondrial alteration that triggers mitochondrion-dependent apoptosis execution and chronology of events remain to be clarified. Some evidence indicates that extrusion of cytochrome c to cytoplasm and the release of apoptogenic molecules precede mPTP opening (21, 22). Other evidence attributes the role of primary event and main switcher of the process to the mPTP opening (19, 20, 23, 24).

The mitochondrial permeability transition pore

A number of different molecules, belonging to both the inner and outer mitochondrial membrane, are dynamically involved in mPTP architecture and orchestrate its regulation (Fig. 3). They include a voltage-dependent anion channel (VDAC), an adenine nucleotide translocase (ANT), and cyclophylin-D (CyP-D) (19, 20, 23). VDAC-ANT-CyP-D complex can recruit a number of other proteins, such as benzodiazepine peripheral receptor, creatine kinase, hexokinase, and Bax. In the model of mPTP opening as the primary event of mitochondrion-dependent apoptosis execution, mPTP opening triggers a proton dissipative current that leads to collapse of transmembrane mitochondrial $\Delta\psi$ and to formation of membrane channels with aspecific leakage of apoptogenic molecules (including cytochrome c). In a very recent model, cytochrome c extrusion is mediated by relocation of cytosolic Bax, an apoptotic member of Bcl-2 family, to the outer mitochondrial membrane and its hetero-oligomerization to form a channel for cytochrome c (24). This phenomenon is accompanied by oligomerization of other apoptotic members of the Bcl-2 family that form other channels by which other apoptogenic proteins are extruded to cytoplasm: they include Apaf-1, Smac/Diablo, AIF, and procaspase 9 (7, 23). Cytochrome c is a double-faced molecule: besides electron transporter inside the energetic mitochondrial respiratory chain, it acquires quite different properties when extruded to cytoplasm. Here, cytochrome c assembles with Apaf-1 and procaspase 9 to form the cell-killer apoptosome.

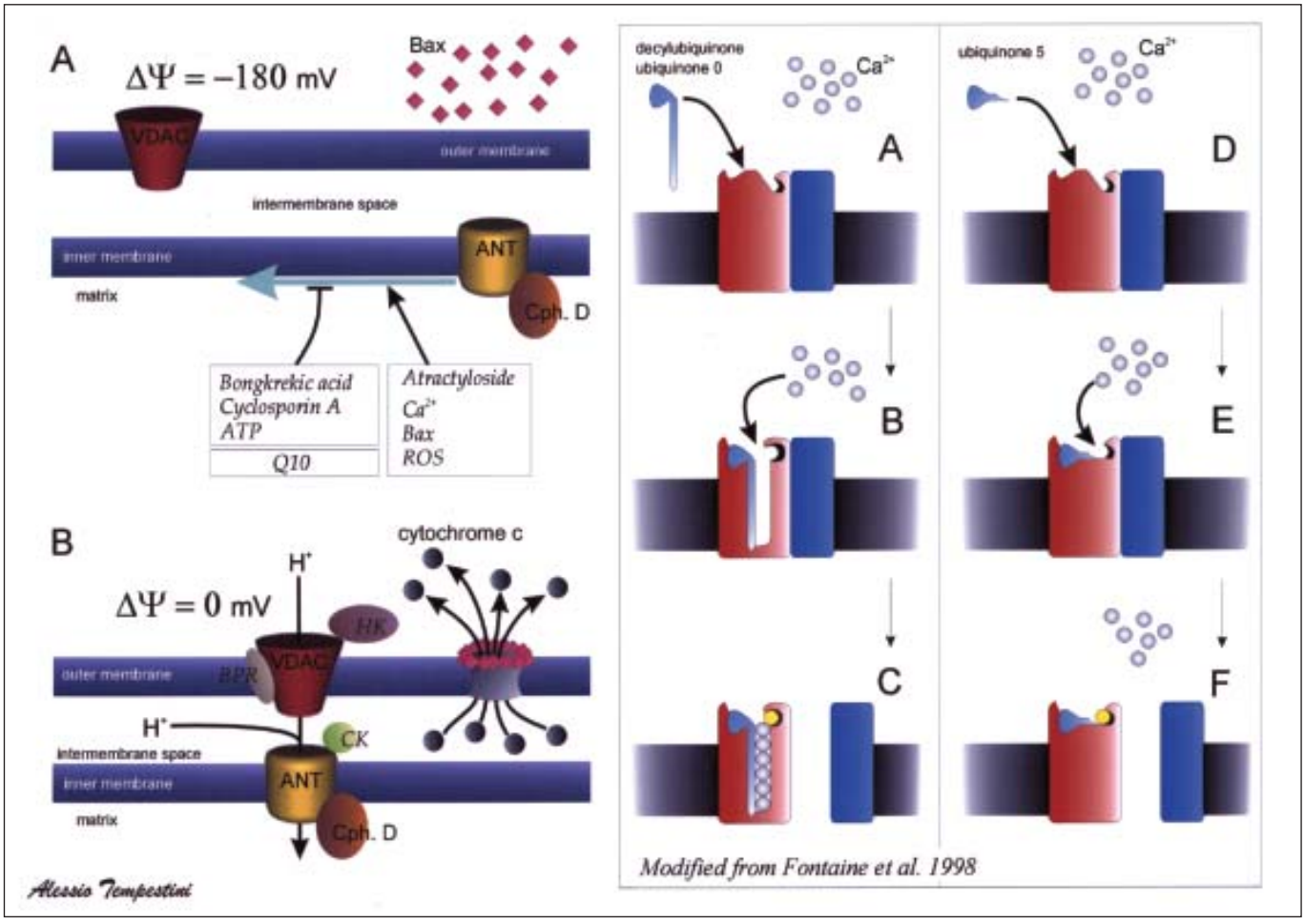

Fig. 3 - *The model of mitochondrial permeability transition pore (mPTP) opening as primary event in apoptosis execution and its ubiquinone-binding site.* **Left:** *The mPTP in its open configuration consists of many components. A voltage dependent anion channel (VDAC) embedded in the outer mitochondrial membrane is in apposition to an adenine nucleotide translocase (ANT) embedded in the inner mitochondrial membrane that binds to a water soluble matrix protein, cyclophylin D. Other mPTP components (BPR, benzodiazepine peripheral receptor; CK, creatine kinase; HK, hexokinase) as well as the different inducers (atractyloside, Ca^{2+}, Bax, reactive oxygen species (ROS)) and inhibitors (cyclosporin A, bongkrekic acid, ATP) of mPTP opening are also shown. Relocation of VDAC and ANT to form a transmembrane channel causes $\Delta\psi$ collapse and conformational alteration of the outer mitochondrial membrane. Consequently, Bax and other apoptotic members of Bcl-2 family are recruited for docking to mitochondrial outer membrane where they oligomerize to form channels by which apoptogenic molecules are released to cytoplasm to trigger procaspase 9 activation-dependent apoptosis execution machinery.* **Right:** *The model of mPTP ubiquinone-binding site designed by Fontaine et al (25) on the basis of the effect on mitochondrial permeability transition of different synthetic ubiquinone analogues applied to isolated mitochondria and modified by authors. The basal condition in the absence of added ubiquinone analogues is shown in* **A** *and* **D**. *When decylubiquinone or ubiquinone 0 enter their ubiquinone-binding site, the accessibility of a specific mPTP Ca^{2+}-binding site is minimal (***B***) and high Ca^{2+} load is required to open mPTP (***C***). Ubiquinone 5 markedly enhances mPTP Ca^{2+}-binding site accessibility (***E***), and relatively low Ca^{2+} load suffices to open mPTP (***F***). Thus, mPTP opening can be achieved either by increasing Ca^{2+} load or by adding ubiquinone 5, which enhances accessibility of the mPTP Ca^{2+}-binding site. CoQ_{10} functions analogically to decylubiquinone in the mPTP. The antiapoptotic effect of CoQ_{10} that we observed in cultured keratocytes treated with free radical independent apoptotic stimuli and demonstrated to be mediated by inhibition of cytochrome c release, procaspase 9 activation, and collapse of mitochondrial $\Delta\psi$ can be explained by this model.*

Fontaine et al (25) have demonstrated in isolated mitochondria that mPTP harbors and is regulated by a ubiquinone-binding site. Investigating the regulation of mPTP by different ubiquinone analogs, they found that both ubiquinone 0 and decylubiquinone inhibited mPTP opening, whereas all other ubiquinones tested (included ubiquinone 5) were ineffective or even antagonistic. All ubiquinones test-

ed except decylubiquinone inhibited the respiratory chain. In agreement with these observations, we have demonstrated in cultured cells that the natural CoQ_{10} is endowed with antiapoptotic properties not only against free radical-dependent (26, 27) but also free radical-non-dependent damaging stimuli (28) and that this effect is mediated by inhibition of cytochrome c extrusion and procaspase 9 activation.

Antiapoptotic signaling pathways

As we have previously mentioned, pro-apoptotic signals are counterbalanced by relevant antiapoptotic signals. Activation of procaspase 3 is prevented by inhibitory apoptosis proteins, which in turn are antagonized by the Smac/Diablo protein released from mitochondria, whereas procaspase 8 activation is prevented by FLIP. Mammalian cells respond to stress by accumulation or activation of highly conserved proteins known as HSP, some of which interfere negatively with apoptosis (10). In particular, HSP-70 is a decoy molecule for Apaf-1, and HSP-27 for cytochrome c: both hinder apoptosome formation and, most probably, procaspase 3 activation as well as the caspase cascade downstream from caspase 3. Furthermore, following identification of an A + U rich element (ARE) of Bcl-2 mRNA that modulates mRNA stability by interacting with AU-binding proteins (29-31), we have observed that HSP-70 also interacts with the Bcl-2 ARE (unpublished data). Preliminary data suggest that HSP-70 stabilizes Bcl-2 mRNA and, therefore, upregulates Bcl-2 gene expression at a post-transcriptional level (Fig. 2).

Disruption of apoptotic program in human diseases

Disruptions of cell proliferation or programmed cell death controls are the main pathogenetic mechanisms in a wide variety of human diseases. Either oncogene- or oncosuppressor gene–related deficiencies of apoptosis are responsible or, at least, co-responsible for neoplastic transformation and progression (32-34). Deficiencies of apoptosis also determine autoimmune diseases (35, 36). On the other hand, enhanced apoptosis is associated with many pathologies, including immunodeficiencies (37); cardiovascular diseases (38-41); neurodegenerative pathologies, such as Creutzfeldt-Jakob (42), Alzheimer (43-45), and Parkinson disease (46); liver (47-50) and renal (51) diseases; and many others. The wide range of ophthalmologic disorders associated with alterations of apoptotic cell-death process, from corneal haze to cataract, from senile maculopathies to glaucoma, is analytically described in another review in this issue (Carella).

Possible pharmacologic repair of apoptosis dysfunctions involving those related to ophthalmologic diseases

The pathogenesis of many human diseases has a contributory or causative altered apoptosis component. Is it possible to repair apoptosis alterations in order to prevent, or to treat, such diseases? The answer is yes – at least, in theory. Apoptotic and antiapoptotic signaling pathways have been studied and so clarified. A variety of molecules involved in apoptosis have been identified as either positive or negative targets for rational therapeutic strategies (48). Many organizations focus on this field in the hope of taking advantage of new discoveries. A variety of new molecular tools are possible candidates for treatment and it is expected that new therapeutics will follow soon. They include death receptor decoys, dominant-negative pseudocaspases, caspase inhibitors, and antisense oligonucleotides modulating apoptosis-related gene expression. In the field of ophthalmologic diseases associated with apoptosis excesses, the authors have designed and patented two therapeutic molecular tools. They are CoQ_{10}, a general antiapoptotic molecule, and antisense oligonucleotides upregulating Bcl-2 expression. CoQ_{10}, in its role as antiapoptotic agent, is suitable both for prevention of haze following corneal keratectomy (26, 27) and for treatment of glaucoma and other retinal diseases (unpublished data). Targeting the Bcl-2 mRNA destabilizing A + U rich element (29) with RNA mimetic (2'-O-methyl) antisense oligonucleotides results in Bcl-2 mRNA stabilization and enhancement of Bcl-2 protein cellular level: such oligonucleotides (ODN) are able to prevent apoptosis in neuronal cells subjected to growth factor starvation (unpublished data). Some apoptosis-modulating therapeutics, including our molecules, are now in human clinical trials or demonstrate efficacy in preclinical animal models.

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Reprint requests to:
Sergio Capaccioli, MD
Department of Experimental Pathology and Oncology
University of Firenze
Viale G.B. Morgagni, 50
50134 Firenze, Italy
sergio@unifi.it

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