Apoptosis and cutaneous melanoma
Ricardo Vieira, Óscar Tellechea and América Figueiredo

Department of Dermatology, Faculty of Medicine, Coimbra University, Coimbra, Portugal.

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ABSTRACT
Apoptosis, or programmed cell death, is a biological process that eliminates unnecessary, virus-infected or mutated cells. In that way, it acts as an antitumour event, preventing the cell immortalization typical of carcinogenesis. Many triggers can induce apoptosis through an intrinsic or an extrinsic pathway, both leading to the activation of a proteolytic cascade, which ends in the degradation of several structural proteins and fragmentation of nuclear DNA.

Proteins from Bcl-2, IAP or p53 families are the most important regulators of apoptosis. A high rate of p53 gene mutations is observed in most human cancers but only in about 10% of all cutaneous melanomas. However, the disturbance of some intracellular signalling pathways that occurs commonly in melanoma is responsible for the down-regulation of p53 protein and also dysfunction of other regulatory proteins (Bcl-2 and IAP families, Smac/DIABLO and Omi/HtrA2). Therefore, apoptosis cascade evasion and consequent abnormal cell survival is a common event in cutaneous melanoma. Apoptosis proteins or genes are possible therapeutic targets to consider in melanoma treatment in the future.

INTRODUCTION
The word apoptosis has its origin in the Greek term αποπτοσις, which means autumn leaves fall. Biologically, apoptosis represents a programmed cell death, a physiological event that leads to a natural selection in a cellular level, promoting the elimination of unnecessary, virus-infected or mutated cells.

Apoptosis is different from necrosis. The cell does not become oedematous and cell content is not released in the interstitial fluid. This kind of cell death depends on the energy provided by adenosine-triphosphate (ATP) molecules. Microscopically, the fragmentation of nuclear envelope and cell membranes is observed, leading to the creation of apoptotic vesicles recognized and phagocytised by macrophages in an inflammation-free event.

Apoptosis has a central role in the course of several biological processes, as organo-
genesis, haematopoietic and epithelial cell renewal, cyclic involution of female reproductive organs, atrophy induced by the absence of growth factors and cell-mediated cytotoxicity. It seems to acquire major relevance in antitumour defence, eliminating transformed cells. Therefore, there are many proto-oncogens or tumour suppressor genes encoding proteins that take part in the apoptotic process. Mutations in critical sites of these genes may affect cell survival, leading to malignant transformation of many cell types, including melanocytes.

**THE CASPASES CASCADE**

Apoptosis is essentially the result of the activation in cascade of several particular proteins named caspases (Fig. 1). The caspases are evolutionarily conserved proteases that mediate apoptosis through aspartate-specific cleavage of a large variety of cellular proteins. They are synthesized as inactive precursors (procaspases) that can be activated sequentially by proteolytic cleavage catalysed by other previously activated caspases. Procaspases activation is a process submitted to a certain hierarchy. There are initiator caspases (caspases 2, 8, 9 and 10) that are activated by several triggers and are able to mediate the activation of effector caspases (caspases 3, 6 and 7).

The final result of the caspases cascade is the digestion of a wide number of structural proteins and the degradation of chromosomal DNA. For instance, caspase 3, after activation by initiator caspases 8 or 9, is able to cleave gelsolin, a protein connected to the actin cytoskeleton and important to the maintenance of cell structure stability. Caspase 3 also cleaves the ICAD protein (inhibitor of caspase-activated DNase), releasing the caspase-activated DNase (CAD) into the nucleus. The result is an internucleosomal fragmentation of nuclear DNA in blocks of about 200 base pairs.

**TRIGGERS OF APOPTOSIS**

Triggers are needed to start apoptosis. They can act through two different pathways (Fig. 1). The transmembrane protein Fas (CD95), the TNFα receptors (TNF-R1 and TRAIL-R2) and enzymes from the lytic granules of cytotoxic T cells (perforin, granzyme A and granzyme B) are the major triggers of an extrinsic pathway started in cell membrane after binding of certain soluble molecules as FasL and TNFα or after an effector cell-mediated immunologic response. A different process (intrinsic pathway) is a consequence of perturbation of the mitochondrial homeostasis, as occurs in cells submitted to growth factors privation or in cells that carry important genomic damage. Proteins of Bcl-2 family or p53 family are major apoptosis triggers through this intrinsic pathway.

**EXTRINSIC PATHWAY**

Independently of the trigger, the first event of the extrinsic pathway is the activation of procaspase 8. The active caspase 8 subsequently activates the effector caspases 3, 6 and 9 (Fig. 1).

When Fas/FasL, TNFα/TNF-R1 and TNFα/TRAIL-R2 complexes act as apoptotic triggers, the activation of procaspase 8 is mediated by a common adaptor molecule named FADD (Fas-associated death domain). The interaction between FADD and Fas/FasL or TNFα/TRAIL-R2 occurs directly. Conversely, TNFα/TNF-R1 com-
plex interacts with FADD using an intermediate protein known as TRADD (TNFR1-associated death domain)\textsuperscript{3,8}.

Finally, FADD and TRADD may also activate the effector caspases through activation of procaspase 2, an event mediated by RIP protein\textsuperscript{4}.

**INTRINSIC PATHWAY**

Cytosolic cytochrome c release from mitochondria is the first step of the intrinsic pathway. This step is the result of a decisive change in mitochondrial outer membrane permeability strictly regulated by Bcl-2 proteins\textsuperscript{5}.
Free cytochrome c in cytosol will interact with Apaf-1 and procaspase 9 to form the apoptosomic complex. The cleavage of procaspase 9 occurs within the apoptosomic complex, leading to the production of active caspase 9. Activation of effector caspases 3, 6 and 7 is the final event mediated by the proteolytic activity of caspase 9 (Fig. 1).

**REGULATION OF APOPTOSIS**

The most important apoptosis regulatory proteins are members of Bcl-2, IAP and p53 families.

a) Bcl-2 family:

Bcl-2 is encoded by a gene located in 18q21 and was first described in follicular B lymphoma cell lines with translocation t(14;18). Beyond Bcl-2 itself, a wide number of Bcl-2 family members were characterized. They can act as proapoptotic (Bax, Bak, Bad, Bik, Bid, Bim, Bod, Bmf, Hrk, Nix, Noxa, PUMA and Bcl-Xs) or antiapoptotic proteins (Bcl-2, Bcl-w, Bcl-XL, Mcl-1 and A1).

All Bcl-2 family members share some common structural characteristics. Apart from Bad and Bid, which are generally found in a soluble form in cytosol, Bcl-2 proteins have a C-terminal hydrophobic domain that gives affinity to the membranes of several organelles, namely outer mitochondrial membrane, endoplasmic reticule and nuclear envelope. They also have diverse combinations of four different domains (BH1, BH2, BH3 and BH4), allowing to classify the Bcl-2 proteins into three distinct groups:

- Group I: antiapoptotic proteins with BH1, BH2, BH3 and BH4 domains (Bcl-2, Bcl-XL, Bcl-w, Mcl-1 and A1).
- Group II: proapoptotic proteins with BH1, BH2 and BH3 domains (Bax, Bak and Bok).
- Group III: proapoptotic proteins with BH3 domain (Bik, Bid, Bad, Hrk, Bim, Bod, Bmf, Nix, Noxa and PUMA). Despite having a BH4 domain, Bcl-XS is commonly included in this group.

The mechanism of action of Bcl-2 proteins is not well understood. It is hypothesized that they can be translocated from mitochondrial intermembrane space to mitochondrial outer membrane when apoptosis is triggered. That mobilization occurs as a result of critical phosphorylations of Bcl-2 proteins mediated by several kinases, namely Akt3. Phosphorylated Bcl-2 molecules are able to form homo or heterodimers, migrating consecutively to mitochondrial outer membrane.

Bcl-2 proteins dimerization can induce proapoptotic or antiapoptotic events. For instance, Bcl-2/Bcl-2 homodimer inhibits cell death. Conversely, the Bax/Bax homodimer or Bax/Bcl-2 and Bad/Bcl-XL heterodimers are proapoptotics. Heterodimers formed by proteins from group III are always proapoptotics.

The ability to produce inhibition or stimulation of apoptosis can be elucidated by different biological function of Bcl-2 proteins:

1. Proapoptotic dimers control cytochrome c, AIF protein (apoptosis inducer factor), Smac/DIABLO and Omi/HtrA2 release from mitochondria. Cytochrome c, as previously seen, is part of the apoptosomic complex. AIF is an apoptosis inducer with unknown mechanism of action. Smac/DIABLO and Omi/HtrA2 are X-IAP
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(X-linked inhibitor of apoptosis) neutralizers.

2. Several antiapoptotic dimers are responsible for Apaf-1 sequestration, blocking the formation of the apoptosomic complex.1

3. Several antiapoptotic dimers create ionic megachannels between inner and outer mitochondrial membrane, abolishing the transmembrane electric potential (proton motive force) leading to the suspension of cell energetic production.10

b) IAP family
The IAP family is composed by several conserved proteins (X-IAP, cIAP1, cIAP2, NAIP, ML-IAP and surviving), with the common capacity of inhibiting apoptosis.11

X-IAP is a potent inhibitor of caspases proteolytic activity. The proteins cIAP1 and cIAP2 are ubiquitin-ligases to Smac/DIABLO, leading to selective proteolysis of that protein.15

c) p53 family
The p53 family includes p53, p63 and p73 proteins.16 All members of this family have a highly conserved domain with affinity to certain DNA sequences. Therefore, all the p53 family members act as transcription factors.

P53 is the well known member of the family and also the well understood proapoptotic protein. This molecule induces expression of a wide number of genes in response to DNA damage. When DNA damage is minimal, p53 blocks cell cycle and stimulates expression of DNA-repairing machinery. P53 can also trigger apoptosis intrinsic pathway when DNA damage is severe and irreversible, leading to the cell sacrifice when critical genomic damage can affect the homeostasis of a living tissue or even a whole organism.17

The activity of p53 interferes with several cellular events as genetic transcription, DNA synthesis and repair, cell cycle, apoptosis and senescence. To perform their actions, p53 binds to the promoter sequences of its target genes with consequent up-regulation of proteins that participate in these biological processes. Many genes are recognized targets of p53, as many cell cycle regulators (p21Cip1, cyclin E and TGFβ), apoptosis regulators (Bax, Bcl-3, Bak, Noxa, PUMA, IAP, Fas) and DNA-repair enzymes (BTG2 and DDB2).

The proapoptotic function of p53 is increased by two proteins known as ASPP1 and ASPP2 (inhibitor of apoptosis-stimulating protein for p53 1 and 2). ASPP1 and ASPP2 are able to bind to the functional domain of p53 increasing its affinity to the promoters of genes encoding important proapoptotic proteins. Independently of p53, ASPP1 and ASPP2 can also induce apoptosis acting by the same way with DNA-binding domains of p63 or p73.18

The IASPP protein (inhibitor of apoptosis-stimulating protein for p53) is a direct inhibitor of p53. MDM2 (mouse double-minute 2 protein) is another important inhibitor of p53, acting indirectly as ubiquitin-ligase that decreases p53 half-life through ubiquitin-dependent proteolysis.19

RELEVANCE IN THE GENESIS OF MELANOMA

Apoptosis malfunction with cell immortalization is a common occurrence in human neoplasms. The p53 protein plays a major role in human carcinogenesis as it is mutated in about 35% to 40% of all human
cancers. These mutations are even more common in skin squamous-cell carcinomas (more than 90%) or in basal-cell carcinomas (about 40% to 50%). However, in cutaneous melanoma, p53 is mutated only in a rate of about 10%. This low rate does not mean that p53 has only a minor role in melanoma carcinogenesis. In fact, inactivation of the p53 protein can result from other events than loss-of-function mutations in the p53 gene. For instance, mutations of the tumour suppressor gene CDKN2A are frequent in cutaneous melanoma (40% in familial melanomas and 10% in sporadic melanomas). This gene encodes two proteins (P14ARF and p16INK4a) mainly responsible for cell cycle regulation. Nevertheless, p14ARF is also the most important inhibitor of MDM2. Its loss involves a subsequent increase in MDM2 function resulting in p53 destruction. In this circumstance, mutations of the p53 gene would result superfluous.

The most common way to induce cell survival in melanoma cells is related with the disturbance of several signalling pathways. As a major example, frequent up-regulation of signalling through Raf/MEK/ERK and PI3K/Akt pathways leads to the inactivation of the proapoptotic Bad protein. This event is a consequence of Bad phosphorylation catalyzed by two signalling kinases, respectively Raf-1 (intermediary of Raf/MEK/ERK signalling pathway) and Akt3 (intermediary of PI3K/Akt signalling pathway). Akt3 also phosphorylate procaspase 9, producing a proteolytic-resistant protein.

The great importance of cell survival in melanoma cells is unquestionable. According to this supposition, some anti-cancer drugs are being developed in order to induce tumour cells to enter in apoptosis. One of these drugs (genasense or oblimersen) is an oligonucleotide antisense sequence that blocks the initiation codon of Bcl-2 mRNA. In vitro studies were very promising and encouraged the performance of a phase III double-blind clinical trial with 771 stage IV melanoma patients, comparing one arm treated with dacarbazine alone with another arm treated with an association of dacarbazine and genasense. The survival was larger in this second arm as was the global response rates (11.7% versus 6.8%). However, the overall survival was not affected by dacarbazine and genasense regimen. In that way, the potentiality of targeting apoptotic proteins to treat cancer is even an attempt to explore in the future.

CONCLUSIONS

Apoptosis dysfunction is apparently a very common event in melanoma as in many human cancers. P53 is generally the most affected tumour suppressor gene related with non-melanoma skin cancer but not with cutaneous melanoma. Despite the low rate of p53 mutations, a functional down-regulation of p53 protein is frequently observed in melanoma cell lines as a consequence of disturbance of some intracellular signalling pathways.

Apoptotic proteins and their genes are possible therapeutic targets in the future. The design of new drugs able to block abnormal molecules will represent an attempt to correct the typical immortality of tumour cells, directly inducing cell death within the tumour.
References