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MELANOMA SURVIVAL STRATEGIES: THE INTRINSIC APOPTOTIC PATHWAY – UPSTREAM AND DOWNSTREAM REGULATORS

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ABSTRACT

Apoptotic cell death is involved in development and tissue homeostasis in numerous organisms, and changes in the apoptotic pathways are associated with many diseases, including cancer. The first evidence for an association between apoptosis and cancer was the discovery that the oncogene *bcl-2* was involved in cell survival in lymphoma. Since then, alterations in the expression of genes that participate in cell survival pathways and resistance to apoptosis have become a hallmark of cancer. A failure to trigger apoptosis properly is an essential requirement during tumor progression and contributes to tumor resistance to radio- or chemotherapy. Melanoma, one of the most aggressive cancers, is characterized by an elevated capacity to metastasize and by a high resistance to drugs. The strategies used by melanoma cells to avoid apoptosis often differ from those in other cancer cells. For example, in contrast to many tumors that frequently show a loss of p53 expression, melanoma maintains p53 expression but alters the p53 pathways. In this review, we summarize various aspects of melanocyte biology and consider the genetic alterations exploited by melanoma cells to escape apoptosis. We also discuss recent findings that have extended our understanding of the resistance of melanocytes to apoptosis during tumor progression.

Key words: Apoptosis resistance, melanoma, tumor progression

INTRODUCTION

Neoplastic transformation is a complex, multi-step process in which cancer cells gain the abilities for uncontrolled proliferation and enhanced survival during tumor development. In 2000, Hanahan and Weinberg [37], in an outstanding review on the core mechanisms of cancer development, defined the evasion of programmed cell death as one of the six capabilities required for tumor cells to survive during tumor progression. Apoptosis is a type of programmed cell death in which either specific extracellular signals or internal stimuli induce an enzymatic cascade orchestrated by cysteine aspartate proteases (caspases) that results in a controlled process of cell destruction characterized by rapid, dramatic morphological changes. Although other processes of programmed cell death have been reported in association with cancer development [9], the role of apoptosis in tumorigenesis is currently the best characterized. In addition to allowing cancer cells to survive all challenging events during oncogenesis, the avoidance of apoptosis also confers therapeutic resistance to tumors [37,46].

The basic molecular mechanisms involved in the induction and execution of apoptosis have been extensively reviewed elsewhere and are beyond the scope of this review [36,45,106]. Briefly, apoptosis can be triggered by two major pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. The extrinsic apoptotic pathway is initiated by the binding of cytokines such as tumor necrosis factor-α (TNF-α), TNF-related apoptosis inducing ligand (TRAIL) and fibroblast associated ligand (FasL) to their receptors, referred to as death receptors. Through a series of adaptor proteins, death receptors trigger the activation of pro-caspase-8, a regulatory caspase that in turn activates the executor caspases (caspases 3, 6 and 7), leading to cell destruction. In the intrinsic apoptotic pathway (Fig. 1), internal stimuli, such as DNA damage, hypoxia, oxidative stress, growth factor deprivation and cell detachment trigger the activation of pro-apoptotic Bcl-2 family members, including Bax/Bak and BH3-only proteins. Interference with the pro-survival Bcl-2 members and/or direct

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activation of Bax/Bak by BH3-only proteins leads to mitochondrial outer membrane permeabilization (MOMP). This permeabilization results in the release of cytochrome-c and other molecules from the mitochondrial intermembrane space.

The release of cytochrome-c is considered to be a central event in the intrinsic apoptotic pathway and a series of anti- or pro-apoptotic proteins of the Bcl-2 family are involved in its regulation. In the cytosol, cytochrome c binds to apoptotic activator factor-1 (Apaf-1) which then oligomerizes to form the apoptosome, a multi-molecular complex that recruits and activates caspase-9; like caspase-8, this caspase activates executor caspases. Both apoptotic pathways converge on the activation of effector caspases and are interconnected. In certain cells, caspase-8, which is activated by death receptors, cleaves Bid, a BH3-only pro-apoptotic protein, promoting its translocation to mitochondria where it induces MOMP.

All cancer cells show a certain resistance to apoptosis, with some types being remarkably resistant to cell death. Melanoma, one of the most aggressive malignancies, is characterized by an elevated capacity to metastasize and by high resistance to drugs (reviewed in [33,38,86]). Although several chemotherapeutic drugs have been tested for the treatment of advanced melanoma, none of them has shown any improvement over the alkylating agent dacarbazine, the only drug approved by the Food and Drug Agency (FDA) as a single agent. Dacarbazine is effective in 10-20% of melanoma cases, with complete remission in only 5% [33,38,86].

Although the frequency of melanoma in Brazil is lower than in the United States, Australia and European countries, the incidence of this disease and its associated mortality have risen progressively in recent years. Thus, the Brazilian National Institute of Cancer estimated that in 2003 there were 4,370 new cases of melanoma whereas in 2006 the number of new cases is estimated to be 5,760 (http//: www.inca.br).

Melanoma often arises from inherited or common acquired nevi, which are pigmentary melanocytic lesions that can progress to dysplastic nevi and may culminate in radial growth phase (RGP) melanoma, an early melanoma lesion that is confined to the epidermis (reviewed in [62]). Preneoplastic and early neoplastic lesions are characterized by double-strand breaks in DNA caused by replication stress during aberrant cell cycles driven by oncogene activation, or by impairment of the cell cycle barrier at the G1/S transition. This breakage leads to activation of the DNA damage checkpoint (including ATM, Chk2 and Cdk1-Y15 phosphorylation) that is associated with cell cycle arrest in the dysplastic lesion, and increased proliferation in the primary melanoma [31]. Thus, a malignant tumor will only arise from cells that are released from the constraints on cell cycle progression. The growth of aberrant cells enhances genetic instability and, together with the suppression of apoptosis, potentiates the malignancy [6,31,95].

A crucial histological alteration seen in the primary tumor during melanoma progression is the transition from an RGP to a vertical growth phase (VGP). In this transition, the cells that were growing only laterally in the epidermis invade the dermis and acquire metastatic potential. In contrast to RGP cells, VGP cells become independent of growth signals from keratinocytes, display anchorage-independent growth and are tumorigenic if inoculated into immunodeficient mice (reviewed in [62]). All of these features indicate that for this transition to proceed the cells probably acquire defects in their apoptotic machinery since growth factor withdrawal and loss of adhesion are pro-apoptotic stimuli for melanocytic cells harboring initial genetic alterations, such as a loss of function in $p16^{Ink4a}$ (a Cdk4a inhibitor with a role in G1 arrest). Despite their extended life span in culture, melanocytic cells lacking $p16^{Ink4a}$ show a high rate of apoptotic death when cultured independently of keratinocytes or growth factors produced by these cells [91].

Clinically, the thickness and ulceration of the primary tumor are considered the major prognostic factors. However, the lack of molecular markers for specific stages of melanoma progression makes prognosis difficult. Gene expression analyses have indicated that melanoma, like other tumors, can be divided into several subtypes based on their molecular profiles that, in some cases, have been related to tumor aggressiveness and patient survival [1,13]. Hence, it may be possible to define more powerful prognostic markers by characterizing the molecular alterations displayed by melanoma cells. In addition, characterization of the main molecular pathways and mechanisms that sustain melanoma survival will probably allow the targeting of specific molecules in order to allow effective therapy.

Instead of acquiring drug resistance as a consequence of treatment pressure, as occurs in other cancers, melanoma has an intrinsic resistance to many anti-cancer drugs. This intrinsic resistance to death

can be partly explained by the biology of melanocytes, the precursor cells of melanoma, that provide protection against UV radiation through the synthesis and distribution of melanin to keratinocytes. As a requirement for these functions, melanocytes have evolved a potent DNA repair system that renders them highly resistant to UV irradiation. These cells are also adapted to oxidative stress resulting from melanogenesis [86], and to hypoxia, a common condition in normal skin [7].

During tumor progression, melanomas accumulate alterations in several genes that encode proteins involved in apoptotic pathways, including the p53 pathways, or those acting in pro-survival pathways, such as Akt/Pi3K, PTEN, B-Raf, NF-κB. Deregulation of pro- and anti-apoptotic members of Bcl2 family and inhibitor of apoptosis proteins (IAP) such as c-FLIP, survivin and livin has also been detected in melanoma. In addition, death receptors such as TRAIL, Fas and TNFR, their ligands, and adaptor proteins such as TRAF2 are targets for dysfunctions (reviewed in [43,44,86]). The inhibition of apoptosis in melanoma, as also in other cancers, can result from the activation of anti-apoptotic factors, downregulation of pro-apoptotic factors, strengthening of cell survival signals and expression of multidrug resistance transporters. However, this inhibition may involve specific targets different from those used in most other cancers. Although recent studies have provided new insights on these targets, there has been a corresponding increase in the complexity of the interactions involved and the role of molecules such as Apaf-1 and MITF in cell transformation and drug resistance remains controversial.

In this review, we summarize recent findings about some of the main molecular mediators involved in the intrinsic apoptotic pathway that have been implicated in the ability of melanoma cells to escape apoptosis. We also discuss the controversial contribution of certain genes implicated in the resistance of melanoma to apoptosis.

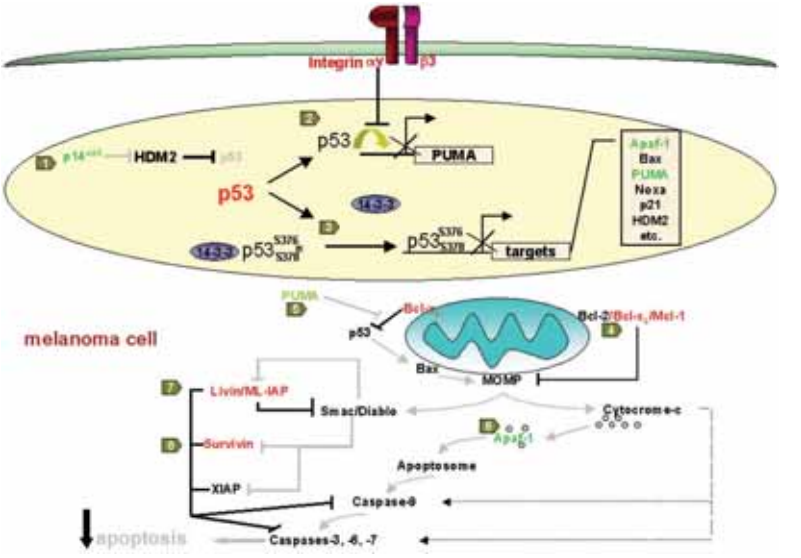

Figure 1. Strategies used by melanoma cells to disrupt the mitochondrial p53-dependent apoptotic pathway. Melanoma cells often lack $p14^{ARF}$ expression, a stabilizer of p53 protein (1). However, instead of expressing reduced p53 levels, melanoma cells frequently display high levels of wild-type p53 protein, indicating that other mechanisms contributing to the disruption of the p53 pathway are involved. The p53-dependent transcription pathway can be altered by expression of αv integrin, leading to downregulation of its DNA-binding activity and a reduced expression of its direct target PUMA (2), or by aberrant phosphorylation of p53 protein, thereby attenuating its binding to 14-3-3 protein and, consequently, its activity in transactivation (3). Melanomas also overexpress the anti-apoptotic proteins Bcl-x_L and Mcl-1, which could increase inhibition of the MOMP in these tumors (4). A possible direct mitochondrial proapoptotic function of p53 may be downregulated by Bcl-x_L overexpression and the reduction in PUMA levels seen in these cells, although this remains to be confirmed (4 and 5). A reduction in or lack of Apaf-1 expression (6), the overexpression of the IAPS ML-IAP (7) and survivin (8) could also contribute to the reduced levels of apoptosis seen in melanoma cells. Green - downregulated molecules, red - upregulated molecules, gray - suppressed molecules or pathways.

What is new about the paradoxical expression of wild type p53 protein in melanoma cells?

The p53 tumor suppressor protein is induced by several types of cellular stress stimuli and plays a fundamental role in the apoptosis triggered by different mechanisms. The intrinsic apoptotic pathway can be regulated by p53 through the transactivation of genes, including Apaf-1, PUMA, Noxa, Bax, and the repression of anti-apoptotic genes such as Bcl-2 and the IAP survivin [46,96]. p53 can also induce the expression of proteins involved in the extrinsic apoptotic pathway, such as the death receptors Fas and TRAIL-R2 [46].

In contrast to most tumors, melanomas have a low frequency of p53 mutations (see [62]). An important factor that regulates p53 activity is $p14^{ARF}$ (alternative reading frame), a protein that stabilizes p53 through inhibition of the E3 ubiquitin ligase HDM2 (MDM2 in mice). $p14^{ARF}$ is coded by a gene that maps to a locus frequently mutated or deleted in melanomas, especially in familial melanoma in which such mutations occur in ~40% of the cases [70]. However, this locus codes for $p16^{INK4a}$ and $p14^{ARF}$ (reviewed in [16]), and although exclusive disruption of $p14^{ARF}$ (mutation on exon 1β) has rarely been described [51,72]), many (nearly half) of the mutations mapped in this locus are present in the common exon 2 and several of them affect the amino acid sequences of $p16^{INK4a}$ and $p14^{ARF}$ [71]. Rizos *et al.* [71] have shown that mutations in exon 2 of this gene impair the cellular localization of $p14^{ARF}$ in human cells.

The relative contribution of $p19^{ARF}$ and $p16^{Ink4a}$ and how they cooperate in tumorigenesis have been studied in transgenic mice [47,50,83]. These studies have shown that $p19^{ARF}$ has a role in melanoma susceptibility, but it is unclear whether mutations affecting this protein in exon 2 play a role in tumorigenesis in humans. Inherited or early preneoplastic mutations in this locus that affect $p16^{Ink4a}$, $p14^{ARF}$ or both gene products could lead to oncogenic-like replication stress or the accumulation of UV light-induced DNA damage because of inefficient repair. Gourgolis *et al.* [31] and Rünger *et al.* [75] have provided evidence to support this suggestion. The findings of these two studies indicate that the intrinsic resistance of melanocytes to apoptosis, in contrast to the progression to carcinoma, mitigates the constraints of p53 on cell survival, resulting in selective pressure for the inactivation of other genes in order to escape cell cycle arrest and allow a progression to melanoma.

Over-expression of wild-type p53 protein is frequently seen in melanoma (reviewed in [43]). p53 activity can be disrupted through defects in other molecules acting upstream or downstream in the p53 pathway. However, the predicted effect of $p14^{ARF}$ dysfunction is a decrease in the level of p53 protein (Fig. 1). Hence, other defects probably contribute to p53 pathway dysfunction in melanoma cells. Satyamoorthy *et al.* [78] reported that some melanoma cells with high levels of wild-type p53 expression have deficient phosphorylation and dephosphorylation of this protein, thereby attenuating the association of p53 with 14-3-3 proteins (Fig. 1). In such cells, the intranuclear localization and the DNA binding capacity of p53 were preserved whereas the positive transcriptional targets of p53 were not induced, even when the cells were infected with an adenoviral vector expressing wild-type p53. In contrast, the expression of an exogenous wild-type p53 in cells with a mutant p53 increased the expression of p53 target genes. Based on these results, the authors argued that a defect in the regulation of p53 phosphorylation and/or dephosphorylation could explain the lack of a functional p53 pathway, even in cells with a high level of the wild-type protein.

Another interesting mechanism involved in the regulation of p53 function during melanoma progression is the integrin signaling pathway. The role of integrin-mediated signaling in tumor progression has been recognized for a long time. This signaling pathway influences cell invasion and migration (reviewed in [41]) as well as apoptosis triggered by cell detachment (anoikis). The involvement of αvβ3 integrin in melanoma progression was first suggested based on the lack of expression of this integrin in normal melanocytes, nevi and first stage melanoma (non-invasive cells from the RGP), in contrast to the remarkably high levels of expression in melanoma from the VGP (invasive cells) and metastases (reviewed in [81]). Later functional studies demonstrated that the forced expression of β3 integrin induced the progression of RGP cells into a VGP phenotype in three-dimensional skin reconstructs [42].

More recent data suggest that the survival and tumorigenic role of αv integrin in melanoma cells is mediated by the inhibition of p53 protein activity [4]. In this study, electrophoretic mobility shift analysis showed a reduced ability of p53 protein to bind to oligonucleotides containing the p53 recognition

site in melanoma cells expressing αv integrin, in contrast to cells that lack expression of this integrin. Interestingly, the effect of αv integrin expression on p53 DNA-binding activity was only seen when cells were grown in a 3D-collagen model, which mimics the tissue architecture. Remarkably, silencing p53 complemented the lack of αv integrin and produced similar levels of survival and tumorigenicity to those in integrin-positive cells. Assessment of the expression of some p53 transcriptional targets revealed reduced levels of PUMA but not of Bax or Apaf-1 in melanoma cells expressing αv integrin, or in cells with p53 knockdown by siRNA in a 3D-collagen model. Thus, in addition to providing a novel element for explaining the deregulated activity of p53 pathway in melanoma progression, the work by Bao and Strömblad [4] highlighted the critical role of the tissue environment on the cellular responses to integrins and indicated the need to consider this aspect in studies on tumor progression.

A novel aspect of p53 function that has recently emerged relates to the ability of this protein to induce apoptosis by direct interaction with Bcl-2 family members in mitochondria (for a recent review see [5]). p53 shows high affinity interaction with $Bclx_L$ in which it displaces BH3-only members or frees Bax, and a direct activation of Bax (without direct interaction). Interaction with $Bclx_L$ may cooperate to promote MOMP [64] or exert a primary inhibitory role on MOMP by sequestering p53 and avoiding Bax activation [18]. In addition, a temperature-sensitive interaction of a polymorphic variant of p53, R72, with Bak has been shown [52]. Melanoma cells are an excellent model for investigating the direct mitochondrial role of p53 and for exploring possible mechanisms of evading the p53-induced mitochondrial apoptotic response. There are at least three reasons for this: (i) melanomas frequently display high levels of p53, (ii) high rates of $Bclx_L$ over-expression have been observed [94] and (iii) reduced levels of PUMA have been detected in melanoma cells [49]. According to a recent report by Chipuk *et al.* [17], PUMA plays a pivotal role in displacing p53 from its complex with $Bclx_L$, thereby sensitizing cells to apoptosis induced by DNA-damaging stimuli. High levels of $Bclx_L$ and a loss of PUMA therefore fit well as events involved in resistance to a putative apoptotic function of p53 in the mitochondria of melanoma cells (Fig. 1).

Is Apaf-1 a tumor suppressor gene?

A high rate of loss of Apaf-1, a transcriptional target of p53 that is a key regulator of apoptosis triggered by the mitochondrial pathway [15,103], has been shown in melanoma [3,65,85]. Soengas *et al.* [84] were the first to show that a lack of Apaf-1 or caspase-9 leads to reduced *c-myc*–induced cell death and enhanced *c-myc* and *ras*-induced transformation in transgenic mouse embryonic fibroblasts (MEFs). Two years latter, the same group [85] showed that 42% of a panel of 24 metastatic melanoma tumors had low or undetectable levels of Apaf-1 mRNA expression and 10 out of 19 cell lines derived from metastatic melanomas were considered negative (<20% of the expression level seen in melanocytes) for Apaf-1 protein expression. In addition, Apaf-1-negative melanoma cells were found to be extremely resistant to adriamycin, a p53-dependent inducer of apoptosis, and the restoration of Apaf-1 expression rescued drug sensitivity. Allelic loss within or near the Apaf-1 locus is frequently linked to melanoma [27,85], with the loss of Apaf-1 expression in melanoma cells occurring through epigenetic silencing by DNA methylation [85].

Several other studies have demonstrated that Apaf-1 expression is reduced in melanoma cells compared with nevi and normal melanocytes, although the correlation between the level of Apaf-1 expression and the stages of melanoma has not been clearly defined (reviewed in [12]). Based on these findings, it has been postulated that Apaf-1 might be a new tumor suppressor gene important for melanoma development, although recent studies have contradicted this view (see below).

Many studies using different cell types, but not melanocytes, derived from Apaf-1 or caspase-9 null mice have addressed the role of these molecules in cell transformation and chemoresistance. In lymphoid cells from caspase-9 and Apaf-1 null mice, but not in mice over-expressing Bcl-2 or lacking Bim, the activation of effector caspases and cell death with morphological characteristics of apoptosis proceeded as in wild-type cells, leading to the suggestion of an apoptosome-independent activation of effector caspases [57]. Scott *et al.* [80] reported that the loss of Apaf-1 or caspase-9 did not promote c-*myc*-induced transformation in MEFs from knockout mice, nor did it influence the development of c-*myc*-induced B-lymphomas in reconstituted, irradiated mice or the sensitivity of lymphoma cells to drugs. However, the involvement of Apaf-1 in the chemoresistance of human B-lymphoma has been described [90].

The role of Apaf-1 as a tumor suppressor gene has been contested based on studies using myeloid

or mast cells derived from Apaf-1$^{-/-}$, caspase-9$^{-/-}$ or caspase-2$^{-/-}$ mice. These cells show a delay in the apoptosis triggered by cytokine withdrawal and an enhanced resistance to cytotoxic drugs, but have no clonogenic survival advantage [26,56]. These results indicate that the requirement for Apaf-1 apparently depends on the specific cellular context. Although in most cases a loss of Apaf-1 did not confer any advantage for cell transformation, murine myeloid and mast cells required Apaf-1 for apoptotic death whereas murine lymphocytes showed no absolute requirement for this protein. Further studies are required to determine whether the delay in apoptosis promoted by a loss of Apaf-1 in a particular cell can, on its own or in association with additional cellular defects, confer some advantage for cell transformation.

The role of Apaf-1 in melanoma cells was recently addressed by Zanon *et al.* [104] who found that although Apaf-1 expression is reduced in most human melanomas, complete loss of this protein is not a frequent event. Apaf-1 was not detected in 1 of 5 melanocyte samples, 6 of 16 primary melanomas, 7 of 61 lymph node metastatic lesions and 2 of 10 subcutaneous metastases. In addition, this group showed that the resistance to several chemotherapeutic drugs (acting through the mitochondrial pathway) was not different in Apaf-1^{-} and Apaf-1^{+} cells. More intriguingly, apoptosis triggered by these drugs in Apaf-1^{-} cells (as in Apaf1^{+} cells) was associated with the activation of caspases-2, -3, -8 and -9. Hence, although melanoma cells show a marked reduction in Apaf-1 expression (for more details, see [12]) and some reports indicate a negative correlation between the level of Apaf-1 protein and progressive stages of the tumor [3,65,85], additional studies are required to clarify the role of Apaf-1 in neoplastic transformation and drug resistance in melanomas and other tumors.

What is the status of survival signaling pathways in melanoma?

Signaling mediated by Akt (or protein kinase B) and mitogen-activated protein (MAP) kinases involves potent survival and proliferative pathways that induce survival genes and inhibit pro-apoptotic molecules. Recent studies have highlighted the importance of these pathways in melanoma development and have provided evidence for a strong link between melanoma and alterations in several genes that play central roles in these pathways (Fig. 2).

Through their binding to receptor tyrosine kinases, growth factors activate phosphatidyl-inositol-3-kinase (PI3K) that converts phosphatidyl-inositol biphosphate (PIP2) into phosphatidyl-inositol triphosphate (PIP3). The protein kinase B or Akt is activated by PIP3 and stimulates proliferation, migration and survival (reviewed in [73]). The Akt family consists of three members (Akt1, Akt2 and Akt3) encoded by different genes. Akt activity results in the expression of pro-survival molecules, such as Bcl-x_L, through activation of the transcription factor NF-κB and the direct inhibition of pro-apoptotic proteins such as BAD and caspase-9 (Fig. 2). An important negative regulator of Akt is the dual lipid and protein phosphatase PTEN (Phosphatase and Tensin homologue on chromosome 10), which controls the cellular levels of PIP2 and PIP3. PTEN is a tumor suppressor gene and studies reporting PTEN gene mutation and its epigenetic silencing suggest that a loss of PTEN function may occur in nearly 60% of melanomas [73]. The activation of Akt as a consequence of PTEN loss contributes to melanoma tumorigenesis by enhancing the apoptotic resistance of melanoma cells [87].

Although alterations in the PTEN gene in melanoma have been described, direct alterations in AKT genes were not reported until 2004, when Stahl *et al.* [88] found that nearly 60% of the melanoma tumors analyzed (18 in a panel of 30 metastatic tumors) had elevated expression of the AKT3 gene. This finding, together with the fact that extra copy numbers of the chromosomic region 1q43-44, which harbors the AKT3 gene, had been reported in melanoma cells lead the authors to suggest that amplification of the AKT3 gene contributed to the deregulation of Akt activity in melanoma cells [88]. In addition, in this same study, Akt3-specific siRNA alone increased the level of apoptosis in melanoma tumors *in vivo*, based on TUNEL assays. The findings of Stahl *et al.* [88] revealed that Akt3 is a target during transformation in melanoma cells and highlighted the importance of the constitutive activation of PIP3/Akt signaling for melanoma resistance to apoptosis. The data on elevated Akt expression in melanoma were recently confirmed and extended through tissue microarrays and immunohistochemical analyses [19]: the strongest phospho-Akt expression was detected in 17% of normal nevi (12 samples), 43% of dysplasic nevi (58 samples), 49% of primary melanomas (170 samples)

and 77% of metastatic melanoma (52 samples). In addition, there was an inverse correlation between strong Akt expression and patient survival.

The Ras-Raf-ERK/MAPK pathway is another important survival and proliferative pathway, the deregulation of which contributes to melanoma progression. Although constitutive activation of Ras has been documented in ~10% of melanoma cases (reviewed in [29]), the most frequent target of this pathway in melanoma is the B-Raf kinase that, in some studies, has been found to have an activating mutation in up to 80% of cutaneous melanoma samples [29]. A systematic cataloging of B-Raf mutational studies in melanoma by Rodolfo *et al.* [74] showed that the most common B-Raf mutation (V600E) has been detected in 50% of more than 1,300 tumor specimens and 250 melanoma cell lines analyzed. B-Raf mutations were also detected in most melanocytic nevi (~68% of the total), although their frequency differed among the histological subtypes of nevi; these mutations were most frequently associated with acquired (junctional, compound, intradermal), congenital and dysplastic nevi, but were rarely detected in Blue and Sptiz nevi [29,74]. The B-Raf gene encodes a serine-threonine kinase that, in a pathway triggered by growth factors, cytokines and mitogens, is activated by Ras and then activates MAP kinase kinase (MEK).

The importance of B-Raf in melanoma was first suggested in a relatively recent study in which a systematic, genome-wide screening for mutations in cancer-related genes detected B-Raf mutations in 66% of human melanoma tumors, despite a lower frequency in most other tumor types analyzed [21]. Additional studies have shown that ERK (the effector of the Ras/Raf/Mek pathway) is constitutively activated in melanoma tumor and cell lines [79,105] and that this activation depends on B-Raf mutation or on an autocrine mechanism of growth factor stimulation since melanoma cells harboring wild-type B-Raf also have constitutively activated ERK [79]. Although the activation of MAPK pathways may require additional events other than a B-Raf mutation, the relevance of activated B-Raf for melanoma survival has been demonstrated. In a recent study, abrogation of this kinase by using an inducible system for the expression of siRNA against B-Raf increased the level of apoptosis in melanoma tumors *in vivo* and unequivocally showed that B-Raf is required for tumor maintenance [40]. In addition, an inhibitor of Raf induced apoptosis in melanoma cell lines harboring a B-Raf mutation, although the effectiveness of this induction was cell-line dependent [67]. Apoptosis triggered by Raf inhibition is apparently caspase-independent since treating cells with the pan-caspase inhibitor Z-VAD-fmk failed to prevent apoptosis. Panka *et al.* [67] also showed that apoptosis induced by Raf inhibition consisted of events that were even independent of MAPK activation, suggesting that the mechanism by which Raf regulates cell survival is complex and requires additional studies to improve our understanding. Although the participation of B-Raf in the onset and progression of melanoma is not clearly understood, the current evidence confirms the importance of this gene in melanoma survival. The high frequency of mutations in melanoma make this gene an important target for anti-cancer therapies, such as gene therapy or blockade by specific kinase-inhibitors.

A major transcription regulator activated by the PI3K/Akt and Ras/Raf/MAPK signaling pathways in melanoma cells is the transcription factor NF-κB [22,23] (Fig. 2). The up-regulation of NF-κB seen in melanoma and other cancers (reviewed in [48]) has led to the hypothesis that enhanced activation

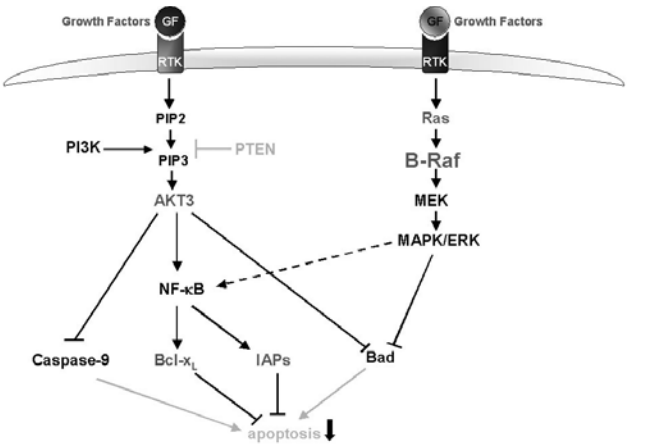

Figure 2. Survival signaling pathways in melanoma. Several positive regulators of survival signaling, such as growth factors, the small GTPase Ras, the kinases Akt3 and B-Raf and the transcription factor NF-κB are up-regulated (black boxes) in melanoma cells, whereas the phosphatase PTEN (gray), a negative regulator of the Akt pathway, is frequently downregulated. These alterations could reinforce the inhibition of pro-apoptotic molecules such as Bad and caspase-9, and the upregulation of anti-apoptotic factors such as IAPs and Bcl-x_L, thereby culminating in reduced apoptotic levels.

of NF-κB is a central mechanism for melanoma development ([60,61,102]; reviewed in [2]). NF-κB is normally involved in pro-inflamatory responses and in the regulation of growth and apoptosis. NF-κB is maintained sequestered in the cytoplasm through binding to IκB, an NF-κB inhibitory factor. Upon appropriate stimulation, IκB is phosphorylated and targeted for proteasomal degradation, thereby allowing NF-κB to translocate into the nucleus to activate target genes. NF-κB induces the expression of several genes that can be divided into four major classes: i) genes involved in the negative regulation of NF-κB, ii) a series of genes involved in immunological responses, iii) anti-apoptotic genes, and iv) pro-proliferation genes. A trimeric complex referred to as the IκB kinase complex (formed by two catalytic subunits, IKKα and IKKβ, and an adaptor protein known as IKKγ or NEMO) is responsible for phosphorylating IκB and targeting it for degradation; hence, the activation of IKK results in the induction of NF-κB activity (reviewed in [55]).

In cancer cells, including melanoma, constitutive activation of the NF-κB pathway is involved in angiogenesis, tumor invasion and the inhibition of apoptosis (reviewed in [2, 48]). NF-κB activity is closely associated with tumor resistance to anti-cancer therapies since TNFα, chemotherapeutic drugs and radiation induce NF-κB activation leading to a decreased effect of these agents [99]. In addition, the down-regulation of NF-κB expression by RNA interference increases the cell sensitivity to drugs [35]. NF-κB induces the expression of several anti-apoptotic factors in melanoma cells, such as cellular IAPs [c-IAP1, c-IAP2, ML-IAP (melanoma IAP) and survivin], Bcl-2-like molecules, including Bcl-x_L and A1, and the TNF-related associated factors TRAF-1, TRAF-2 and c-FLIP, which act as inhibitors in the extrinsic apoptotic pathway. NF-κB also induces the expression of cyclin D1 and Cdk2 which allow escape from cell-cycle control and stimulate proliferation [2,48].

Is MITF an oncogene involved in melanoma progression?

MITF (*Microphthalmia*-associated transcription factor) is a central regulator involved in melanocyte differentiation and pigmentation. MITF is essential for melanocyte differentiation and survival since mutations that inactivate MITF lead to loss of the melanocytic lineage (reviewed in [89]). Bcl-2 is a direct downstream target of MITF that is essential for melanocyte survival [89]. However, Bcl-2 is probably not the only pro-survival MITF effector since clonogenic survival was abrogated in melanoma cells with MITF disruption, even when overexpressing Bcl-2 [59].

In contrast to other melanocytic markers, MITF expression is maintained in nearly all melanomas. Despite the well-defined, central role of MITF in melanocytic differentiation and survival, its participation in the development of melanoma is controversial. Some studies have indicated that reduced MITF expression is associated with melanoma progression [76,82]. In addition, recent results have shown that MITF activates the transcription of the cell-cycle regulators p16/INK4a [54] and $p21^{cip1}$ /*CDKN1A* [14] and can induce cell-cycle arrest in melanocytes and melanoma cells in a p16- or p21-dependent manner. Moreover, in B-Raf-transformed melanocytes, MITF expression is down-regulated, and restoration of the MITF levels in these cells leads to a decreased capacity for colony formation [100]. As expected, the effect of MITF on cell colony number is not mediated by the induction of apoptosis (unlike other oncogenes), but rather caused by the inhibition of proliferation. On the other hand, indications that MITF may have an oncogenic role in melanoma progression have also recently emerged. In a genome-wide analysis aimed at identifying alterations in chromosomal copy number in cancer, the *MITF* gene was found to be amplified in melanoma cell lines and melanoma tumors [28]. The frequency of *MITF* locus amplification was positively correlated with the stage of melanoma progression and was associated with decreased patient survival. All of the melanoma cell lines that showed *MITF* amplification also harbored mutations for activation of the B-Raf pathway and inactivation of the p16 pathway. In addition, in melanocytes genetically modified to inactivate the p53 and p16/Rb pathways, the over-expression of MITF together with mutated B-Raf was sufficient to transform the cells. The influence of MITF on cell survival is also demonstrated by the finding that the expression of a dominant negative form of MITF renders cells more sensitive to growth inhibition by cisplatin and docetaxel. *Hif1a* has been characterized as a new transcriptional target activated by MITF in melanocytes and melanoma cells [10]. *Hif1a* encodes

the hypoxia-induced factor 1, a transcriptional factor involved in oxygen homeostasis that controls the expression of genes associated with several aspects of cancer, including resistance to apoptosis. In a very recent and interesting study, McGill *et al.* [58] demonstrated that MITF is activated by and induces the expression of the c-Met receptor tyrosine kinase, in a positive regulatory loop. The activation of MITF occurred via MAPK phosphorylation triggered by stimulation of the c-Met receptor by hepatocyte growth factor (HGF); phosphorylated MITF then directly targeted the c-Met gene promoter and, within 2 h of stimulation, phospho-MITF was also targeted to proteasome- dependent degradation. The HGF/c-Met pathway has been implicated in growth and invasion in several tumor cell types, including melanoma.

As indicated by Merlino [63], it is counter-intuitive to think that a gene involved in cell cycle arrest could function as an oncogene. Merlino suggests that the loss of $p16^{ink4a}$ could be a mechanism for uncoupling the anti-proliferative and pro-survival activities of MITF. Since MITF amplification has been detected only in cells with a p16 pathway dysfunction [28], the resulting transformation by MITF overexpression in association with B-Raf mutation could depend on the status of p16. However, Wellbrock *et al.* [100] reported an inhibitory effect of MITF overexpression on the proliferation of melanocytes transformed by the expression of a mutant B-Raf in a murine cell line (melan-a) also lacking functional p16 [92,101] Thus, additional defects are probably required to change the antiproliferative activity of MITF into one capable of transforming cells (Fig. 3). The link for this switch may reside in pathways controlled by the c-Met receptor or the transcriptional factor *Hif1a*. In addition, the status of the proteasome-dependent degradation pathway might be fundamental in balancing MITF activities and in driving the cell towards growth or arrest. Future studies will need to address the roles of molecules such as Cdk4/6-cyclin D and the small Cdk inhibitors $p21^{cip1}$ or $p27^{kip1}$. The dual function of the latter two proteins on cell cycle progression, in which they activate Cdk4/6-cyclin D and inhibit Cdk2-cyclin E, may be central to understanding when MITF activation will result in progression through G1 or arrest.

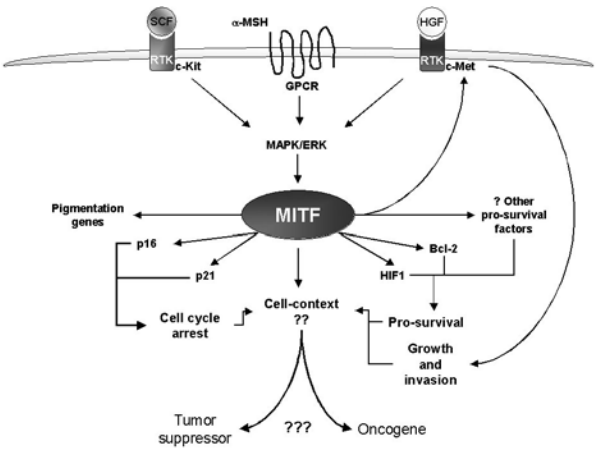

Figure 3. Is MITF an oncogene or a tumor-suppressor gene? MITF is an essential molecule for melanocyte differentiation and survival, but its role in the progression of melanoma is complex and controversial. MITF activates the transcription of genes involved in cell cycle arrest, such as p16 and p21, genes involved in cell survival, such as Bcl-2 and HIF-1, and genes involved in melanoma growth and invasion, such as the receptor tyrosine kinase c-Met. Opposite effects (pro-transformational and anti-proliferative) have been demonstrated for MITF expression in melanoma cells (see text for details). Hence, the cellular context in which the complex and diverse roles of MITF are integrated will determine the final effect of this protein on tumorigenesis.

What anti-apoptotic factors help melanomas evade apoptosis?

The studies discussed above indicate that melanoma cells have several strategies for up-regulating anti-apoptotic molecules and reducing or inactivating pro-apoptotic ones. For pro-apoptotic factors such as members of the Bcl-2 family there is limited and still inconclusive data for their role in melanoma; in contrast, a greater number of studies has addressed the status of anti-apoptotic factors in melanoma progression (reviewed in [11]). There is no clear association between the level of Bcl-2 expression and melanoma progression since some studies have detected an increase in Bcl-2 expression during melanoma development whereas others have reported an opposite change. Nevertheless, many functional studies using gene interference strategies have indicated the importance of Bcl-2 for melanoma cell survival. Unlike Bcl-2, the other two pro-survival members of the Bcl-2 family, Bcl-x_L and MCL-1, have been found to be more consistently associated with melanoma resistance to apoptosis (see [11]).

Other important negative regulators of apoptosis are IAPs (reviewed in [77]). IAPs play an anti-apoptotic function by inhibiting caspases and their

activity is blocked by Smac/Diablo, a protein also released from mitochondria after apoptotic stimuli. Recent studies have raised the possibility that some IAPs might suppress apoptosis through binding to and inhibiting Smac/Diablo [25], thus indicating that the mechanism of IAP action is not simple. Two important IAPs associated with melanoma are survivin and ML-IAP (melanoma-IAP). Survivin is not expressed in normal melanocytes, but is detected in melanocytic nevi and metastatic melanomas [34]. Survivin inhibits apoptosis by caspase-dependent and independent pathways, and plays a role in the mitotic spindle checkpoint [53]. Functional studies have shown that survivin contributes to increased apoptosis resistance in melanoma cells [68,69]. Recent studies have indicated that survivin is a prognostic factor for melanomas since melanomas with higher levels of survivin expression are more resistant to therapeutic agents [93]. Ding *et al.* [24] recently reported the nuclear localization of survivin in a series of melanoma samples, but not in nevi, a result that may contribute for the histopathological staging of melanoma. A nuclear localization of survivin has also been described in other types of tumors, some of which were related to poor prognosis. The mechanisms controling the localization of survivin are poorly understood. Since survivin is preferentially expressed in tumor cells, it probably represents a potential target for anti-cancer therapies. Clearly, further investigation into the roles of survivin and its regulation in melanoma and other cancers is required.

Livin/ML-IAP/KIAP is another IAP that was discovered independently by different groups. ML-IAP (melanoma-IAP) is highly expressed in melanoma cell lines [98], and a recent study using archived melanoma tissues showed that ML-IAP is expressed in 20% of nevus samples and in up to 70% of melanoma samples, although there was no significant difference between primary and metastatic melanomas [30]. The role of ML-IAP in apoptosis and melanoma is still poorly understood, although this molecule is apparently under complex regulation. Nachmias *et al.* [66] demonstrated that livin/ML-IAP is a target for cleavage by caspase-3 and -7 to yield a fragment with pro-apoptotic activity. The potent action of ML-IAP as an anti-apoptotic factor may not depend only on direct caspase inhibition, but also on its capacity to bind and sequester Smac/Diablo, thereby blocking its inhibitory effect on XIAP [97].

The results discussed above illustrate the complexity of IAP function and regulation, as also seen for other regulators of apoptosis. Although many studies have helped to elucidate the role of apoptotic factors in regulating apoptosis and in the ability of tumors to escape apoptosis, many more studies will be required to provide a better understanding of the molecular players involved and to allow the design of specific and potent anti-tumor drugs.

CONCLUSIONS

The resistance of melanomas to apoptosis is a challenge to be overcome by patients, clinicians and scientists. Many genes that contribute to the suppression of apoptosis in melanoma cells have been recognized, and many others remain to be discovered. In this review, we have focused on new information about some of the well-known genes and pathways widely implicated in the resistance of melanomas to programmed death. Results obtained in the last three years have raised new possibilities regarding the function and regulation of these genes. However, the new data also indicate that most molecules are engaged in complex circuits that are still poorly characterized. The function of a specific molecule apparently depends on the contextual aspects of the cells and tissues. Many questions such as Can Apaf-1 act as a tumor suppressor gene? Is MITF a melanocytic-specific oncogene? and Is B-Raf a relevant target for anti-melanoma therapy? will only be answered when key molecular pathways have been well dissected. Other questions will need to consider the tissue context and the relationship between the tumor and its neighboring cells. As discussed above, the ability of αv integrin to regulate p53 activity is only appreciated in conditions that mimic a natural tissue architecture, such as found in 3D-collagen models. Another important aspect is the redundancy of and the interplay among anti-apoptotic pathways, as exemplified by the repression of Bad by Akt and *MAPK* pathways (Fig. 2). This interplay highlights the importance of developing combined therapies to allow treatment via different specific targets.

Despite this complexity, one thing is clear: evasion of apoptosis is one of the most important factors in permitting the progression of cancer. Green and Evan [32] have proposed that uncontrolled proliferation and the suppression of apoptosis form the basic platform necessary for all tumor development

since the other capabilities acquired by tumors are a consequence of deregulated proliferation. Cancer cells need to meet both conditions simultaneously since many, if not all, molecular pathways that trigger proliferation are coupled to mechanisms that induce or sensitize the cells to apoptosis. A remarkable example is the classic oncogene MYC that, in addition to its proliferative effect, also sensitizes the cell to several pro-apoptotic stimuli such as nutrient deprivation, hypoxia and DNA damage. The pro-apoptotic action of MYC is achieved through activation of the ARF/p53 pathway and activation of Bim in a p53-independent manner. (reviewed in [8,20])**.** Suppression of either the p53 pathway or Bim activity is sufficient to impair the pro-apoptotic action of MYC, thereby allowing proliferation and rendering the cells susceptible to transformation. Interestingly, point mutations in MYC in Burkitts´s lymphoma cells can specifically abolish the ability of the mutant Myc protein to activate Bim. Since this mechanism uncouples proliferative and pro-apoptotic actions through an alteration in a single gene, the mutated MYC induces tumorigenesis much more efficiently than does overexpression of the wild-type gene [39] (see [8,20]). Since the mechanisms that allow cells to suppress apoptosis are so essential for tumor development and since the repertoire exploited by tumor cells to avoid apoptosis can be extremely diverse, understanding the mechanisms used by melanoma cells to evade apoptosis is fundamental for developing efficient therapies to combat the resistance of melanomas to programmed death.

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