

Down-Regulation of Mcl-1 by Small Interfering RNA Sensitizes Resistant Melanoma Cells to Fas-Mediated Apoptosis

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Abstract

Resistance of malignant melanoma cells to Fas-mediated apoptosis is among the mechanisms by which they escape immune surveillance. However, the mechanisms contributing to their resistance are not completely understood, and it is still unclear whether antiapoptotic Bcl-2–related family proteins play a role in this resistance. In this study, we report that treatment of Fas-resistant melanoma cell lines with cycloheximide, a general inhibitor of *de novo* protein synthesis, sensitizes them to anti-Fas monoclonal antibody (mAb)–induced apoptosis. The cycloheximide-induced sensitization to Fas-induced apoptosis is associated with a rapid down-regulation of Mcl-1 protein levels, but not that of Bcl-2 or Bcl-xL. Targeting Mcl-1 in these melanoma cell lines with specific small interfering RNA was sufficient to sensitize them to both anti-Fas mAb-induced apoptosis and activation of caspase-9. Furthermore, ectopic expression of Mcl-1 in a Fas-sensitive melanoma cell line rescues the cells from Fas-mediated apoptosis. Our results further show that the expression of Mcl-1 in melanoma cells is regulated by the mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) and not by phosphatidylinositol 3-kinase/AKT signaling pathway. Inhibition of ERK signaling with the mitogen-activated protein/ERK kinase-1 inhibitor or by expressing a dominant negative form of mitogen-activated protein/ERK kinase-1 also sensitizes resistant melanoma cells

to anti-Fas mAb-induced apoptosis. Thus, our study identifies mitogen-activated protein kinase/ERK/Mcl-1 as an important survival signaling pathway in the resistance of melanoma cells to Fas-mediated apoptosis and suggests that its targeting may contribute to the elimination of melanoma tumors by the immune system. (Mol Cancer Res 2008;6(1):42–52)

Introduction

Melanomas are malignant tumor cells derived from pigment-producing melanocytes and are the only cutaneous cancer cells that metastasize and cause death (1). Metastatic melanomas are difficult to treat and respond poorly to treatment by chemotherapy, radiotherapy, and immunotherapy (2, 3). One mechanism that can account for resistance to such treatments is the ability of tumors to escape apoptosis (4). Apoptosis or programmed cell death plays a major role in development, homeostasis, and protection from cancer, and it is now recognized that resistance to apoptosis is a critical factor in tumor progression and malignancy. Apoptotic indices are typically low in melanoma tumors, particularly in advanced stages (5, 6). Thus, understanding the mechanisms by which melanomas resist apoptosis may be beneficial in the design of novel treatments.

Fas (APO-1/CD95)–induced apoptosis is a major pathway in cell-mediated immune response against virus-infected and tumor cells (7–10) and is triggered by Fas ligand, a cytokine that belongs to the tumor necrosis factor superfamily. Stimulation of Fas receptor leads to formation of the death-inducing signaling complex and to the subsequent activation of caspase-8, which, in type I cells, leads directly to activation of effector caspases. In type II cells, however, caspase-8 activation is weak, and the apoptotic signal is amplified by the activation of the mitochondrial apoptotic pathway (11, 12). In these cells, caspase-8 cleaves the proapoptotic Bcl-2 member Bid to generate truncated Bid, which translocates from cytoplasm to the mitochondria, to contribute to the mitochondrial damage, leading to the release of apoptogenic proteins, such as cytochrome *c*. Once released from mitochondria, cytochrome *c* allows the formation of the apoptosome complex and the subsequent activation of caspase-9, which in turn amplifies the caspase-8 signal and leads to the activation of executioner caspases (11, 12).

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Fas receptor signaling seems to serve as a primary death cascade in human melanomas (13). Resistance of melanoma tumors to death induced by Fas ligand is among the mechanisms by which they escape the attacks from immune cells (8, 14) and may also render adaptive immunotherapeutic protocols less efficient (8, 15, 16). It can also be associated with their resistance to apoptosis induced by chemical and physical stimuli as well (17-19). Altered expression of Fas or Fas ligand in aggressive melanoma can partially explain their resistance to Fas-mediated death (4, 17, 20, 21). However, a full understanding of the mechanisms responsible for this resistance is needed for efficient elimination of malignant melanoma cells. Most melanoma cells are considered type II cells and, therefore, depend on the mitochondria death pathway for the execution of Fas-induced apoptosis (6, 22). The mitochondria death pathway is tightly regulated by Bcl-2 family of proteins that comprises both proapoptotic proteins, such as Bax, Bak, and Bid, and antiapoptotic proteins, such as Bcl-2, Mcl-1, and Bcl-xL. Resistance of melanoma cells to Fas-induced apoptosis can be manifested at the level of mitochondria and has been correlated with a defect in cytochrome *c* release (23) and with the expression level of proapoptotic and antiapoptotic Bcl-2-related proteins (24-26). However, most of these studies did not identify conclusively which Bcl-2 member is crucial in melanoma resistance to Fas-induced apoptosis, and the intracellular signaling pathways that regulate the expression of Bcl-2 proteins in these tumors have not been fully investigated.

In this study, we show that the antiapoptotic member of the Bcl-2 family Mcl-1 significantly contributes to the resistance of melanoma cells to Fas-induced apoptosis. Our results show that targeting Mcl-1 by specific small interfering RNA (siRNA) remarkably decreases its expression in resistant melanoma cells and sensitizes them to Fas-induced apoptosis. We further show that the expression of Mcl-1 in melanoma cells is regulated by the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway. Thus, our study identifies the MAPK/ERK/Mcl-1 signaling pathway as an important element of melanoma resistance to Fas-induced apoptosis.

Results

Sensitization of Melanoma Cell Lines to Fas-Mediated Apoptosis by Cycloheximide

Most of melanoma cell lines are resistant to Fas-induced apoptosis. As shown in Fig. 1A, Mel-1 and HT-144 show little apoptosis after treatment with the agonistic anti-Fas monoclonal antibody (mAb) CH11, whereas the M74 melanoma cell line shows significant apoptosis upon Fas activation. Increasing the concentration of anti-Fas mAb or extending the time of treatment did not result in further apoptosis in the resistant melanoma cell lines (data not shown). The expression of Fas receptor is comparable among the three different cell lines (Fig. 1B), suggesting that the resistance of Mel-1 and HT-144 cells to Fas-induced apoptosis may rather occur at the level of Fas-induced intracellular signaling. Accordingly, we hypothesized that up-regulation of antiapoptotic proteins in resistant melanoma cell lines can account for their resistance to Fas-induced apoptosis. Therefore, we tested if cycloheximide, a

general inhibitor of *de novo* protein synthesis, can sensitize resistant cells to Fas-induced apoptosis. Assessment of cell death by flow cytometry for cells in the sub-G₀-G₁ region (DNA content lower than 2N) revealed that treatment of Mel-1 cells with cycloheximide for 2 h before their treatment with anti-Fas mAb CH11 significantly increased their apoptosis up to 30% and 50% after 12 and 24 h of treatment, respectively (Fig. 2A). Simultaneous treatment of Mel-1 cells with cycloheximide and anti-Fas mAb, but not with cycloheximide or CH11 alone, resulted in the activation of apoptotic events associated with the Fas death pathway in melanoma cells (type II), activation of caspase-8, cleavage of Bid, and activation of caspase-9 and caspase-3, as depicted in Fig. 2B. We also verified that Mel-1 cells are type II cells, as caspase-9 inhibitor (Z-LEHD-FMK) significantly reduces their apoptosis in response to anti-Fas mAb and cycloheximide (data not shown). Similarly, pretreatment of resistant HT-144 cells with cycloheximide sensitized them to Fas-mediated apoptosis (Fig. 2C), which is also accompanied by the activation of caspases (data not shown). Together, these results suggest the implication of antiapoptotic proteins in the resistance of melanoma cells to Fas-mediated apoptosis.

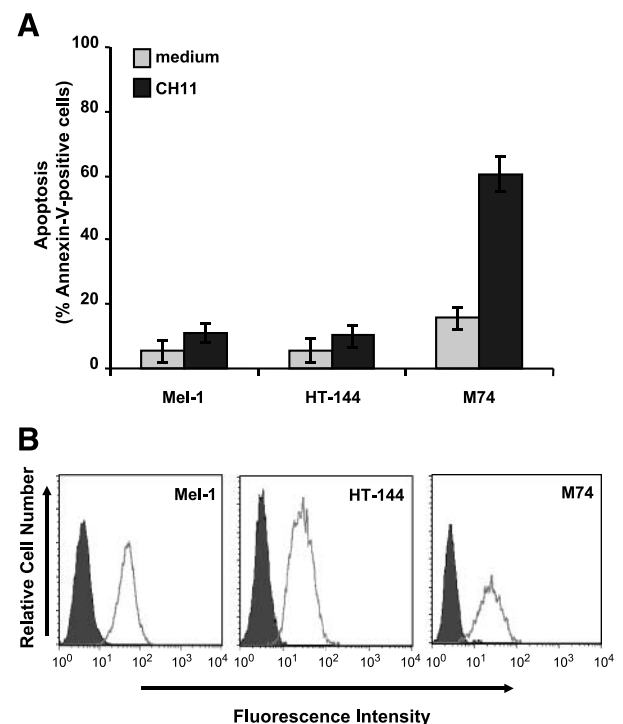


FIGURE 1. Sensitivity of melanoma cell lines to Fas-induced apoptosis. **A.** Melanoma cell lines Mel-1, HT-144, and M74 were treated for 24 h with 500 ng/mL of anti-Fas mAb CH11. Apoptosis was determined using Annexin V/7-AAD staining and flow cytometry analysis. Columns, mean percentages of Annexin-V-positive cells from three independent experiments done in triplicate; bars, SEs. **B.** Expression of Fas receptor on melanoma cell lines. The cells were stained with FITC-conjugated anti-Fas antibody or with isotype-matched control IgG. The cells were then analyzed by flow cytometry using a FACScan, and the data are presented as the peak fluorescence intensity (log scale) of cells stained with anti-Fas mAb (light histograms) and with isotype-matched control mAb (dark histograms).

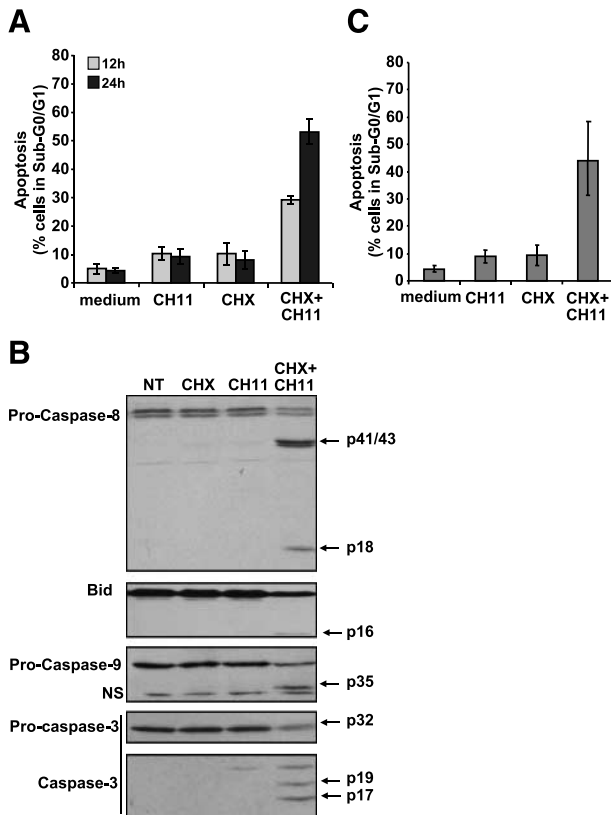


FIGURE 2. Sensitization of melanoma cell lines to Fas-induced apoptosis by cycloheximide (CHX). **A.** Mel-1 cells were treated or not for 2 h with cycloheximide (2 μ g/mL), followed by a treatment with 500 ng/mL of anti-Fas mAb CH11 for 12 or 24 h. Apoptosis was determined by nuclear DNA content and flow cytometry analysis. Columns, mean percentages of cells in the sub- G_0 - G_1 region from three independent experiments done in triplicate; bars, SEs. **B.** Mel-1 cells were treated or not with cycloheximide for 2 h then treated with anti-Fas mAb CH11 for an additional 8 h as indicated. Whole-cell lysates were prepared, and activation of caspase-8, caspase-9, and caspase-3 and cleavage of Bid were determined by immunoblot analysis using specific antibodies as described under Materials and Methods. Arrows, processed forms of Bid and caspases. Results are representative of three independent experiments. NS, nonspecific. **C.** HT-144 cells were treated as described for Mel-1 with cycloheximide, anti-Fas mAb CH11, or both, and apoptosis was determined 24 h later by nuclear DNA content and flow cytometry analysis. The results are presented as in **A**.

The Bcl-2-Related Protein Mcl-1 is Down-Regulated by Cycloheximide in Melanoma Cells

Previous studies have suggested that blockade of the mitochondria death pathway could be important in the resistance of melanoma to death receptor-induced apoptosis (23, 27, 28). Bcl-2-related antiapoptotic proteins are known to regulate the function of the mitochondria and, therefore, can account for the resistance of melanoma cells to Fas-mediated apoptosis. We examined the expression of Bcl-2, Bcl-xL, and Mcl-1 antiapoptotic proteins in Fas-resistant Mel-1 and in HT-144 cells. Treatment of Mel-1 (Fig. 3A) or HT-144 (Fig. 3B) cells with cycloheximide resulted in a rapid down-regulation of Mcl-1, which occurs after 2 h, but had no effect on the levels of Bcl-2. Treatment with cycloheximide also had no effect on the levels of Bcl-xL in Mel-1 cells, but had very slight effect on HT-144 cells after 12 h. As a corollary, Mcl-1

is not detected in the Fas-sensitive M74 cells (Fig. 3C). Together, these results suggest that Mcl-1 is a good candidate in mediating the resistance of melanoma cell lines to Fas-induced apoptosis.

Knockdown of Mcl-1 by siRNA Sensitizes Melanoma Cells to Fas-Mediated Apoptosis

To determine whether the reduction of Mcl-1 observed after cycloheximide treatment is involved in the sensitization of the cells to Fas-induced apoptosis, we used RNA interference strategy to knockdown the expression of Mcl-1 in Fas-resistant melanoma cells and then tested their sensitivity toward Fas-induced apoptosis. As shown in Fig. 4A, transfection of Mel-1 cells with Mcl-1-specific siRNA, but not with control siRNA (a random scrambled sequence), significantly reduces Mcl-1 protein levels by 75% but had no effect on the Bcl-2 or β -actin protein levels. We then analyzed if cells with reduced Mcl-1 become sensitive to apoptosis induced upon Fas triggering. Transfection of Mcl-1 siRNA caused an increase in the spontaneous apoptosis of Mel-1 cells (up to 15%), whereas control siRNA had no effect (Fig. 4B). Addition of anti-Fas mAb CH11 further increases apoptosis up to 30% in Mcl-1 siRNA-treated cells (Fig. 4B) but had no pronounced effect on apoptosis of cells transfected with control siRNA compared with nontransfected control cells (medium; Fig. 4B). To confirm the function of Mcl-1 in the resistance of melanoma cells to Fas-induced apoptosis, we examined the activation of caspase-9, a key apoptotic event downstream of mitochondria in the Fas-signaling cascade. Exposure to Mcl-1 siRNA induced a slight activation of caspase-9 as determined by the cleavage of proenzyme and the appearance of the activated form of caspase-9 (Fig. 4C). Triggering of Mcl-1 siRNA-transfected, but not control

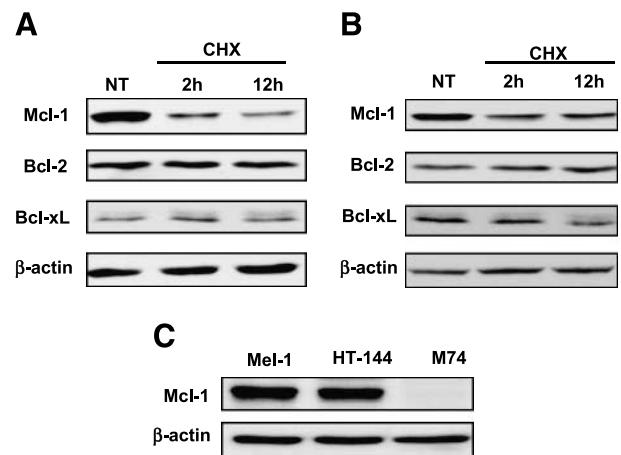


FIGURE 3. Cycloheximide treatment rapidly decreases Mcl-1 protein levels in Fas-resistant melanoma cells. Mel-1 (**A**) and HT-144 (**B**) cells were incubated in the presence of cycloheximide (2 μ g/mL) for the indicated periods of time. Whole-cell extracts were prepared and subjected to immunoblot analysis with specific antibodies against Mcl-1, Bcl-2, or Bcl-xL. **C.** Differential expression of Mcl-1 protein in melanoma cell lines. Whole-cell extracts were prepared from Mel-1, HT-144, and M74 melanoma cell lines, and the expression of Mcl-1 was determined by immunoblot analysis. The blots were stripped and reprobed with anti- β -actin mAb to ensure equal loading. The results are representative of three independent experiments.

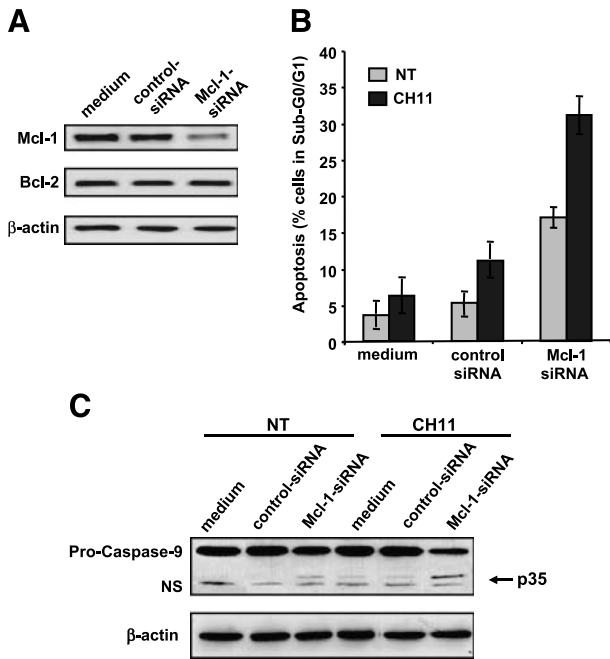


Figure 4. Knockdown of Mcl-1 gene sensitizes Mel-1 cells to Fas-induced apoptosis. **A.** Mcl-1-specific siRNA reduces the expression of Mcl-1 protein. Mcl-1-specific siRNA or negative control siRNA were introduced into the cells using Oligofectamine transfection reagent. The cells were harvested after 48 h, and whole-cell lysates were prepared and subjected to immunoblot analysis using Mcl-1, Bcl-2, and β -actin-specific antibodies. **B.** Mel-1 cells were transfected or not (medium) with control siRNA or Mcl-1-specific siRNA. At 48 h after transfection, the cells were treated or not with anti-Fas mAb CH11 (500 ng/mL, 24 h), and their apoptosis was determined by nuclear DNA content and FACS analysis. Columns, mean percentages of cells in sub-G₀-G₁ region from three independent experiments done in triplicates; bars, SEs. **C.** The cells were transfected and treated as in **B.** After 8 h, whole-cell extracts were prepared, and caspase-9 activation was determined by immunoblot analysis. The blot was stripped and reprobed with anti- β -actin mAb for equal loading. Arrow, processed forms of caspase-9; NS, nonspecific. The results are representative of three different experiments.

siRNA-transfected, Mel-1 cells with anti-Fas mAb CH11 resulted in a marked activation of this caspase (Fig. 4C), indicating that Mcl-1 down-regulation is involved in the activation of caspase-9 downstream of Fas signaling in Mel-1 cells. Knockdown of Mcl-1 also sensitizes other resistant melanoma cell lines, HT-144 and SLM-8, to Fas-induced apoptosis (Fig. 5). To further assess the role of Mcl-1 in the regulation of Fas-induced apoptosis, we have determined if overexpression of wild-type Mcl-1 could rescue the Fas-sensitive, Mcl-1-negative M74 cells from Fas-induced apoptosis. As shown in Fig. 6A, transfection of the cells with a Mcl-1 plasmid led to a significant increase of Mcl-1 protein level without any effect on Bcl-2 level. Furthermore, overexpression of Mcl-1 reduces Fas-induced apoptosis by 50% (Fig. 6B). In contrast, cells transfected with a control plasmid did not affect Mcl-1 level and did not protect the cells from Fas-induced apoptosis (Fig. 6). Together, these results indicate that Mcl-1 is a major Bcl-2 antiapoptotic protein that confers resistance of melanoma cells to Fas-induced apoptosis.

Knockdown of Bcl-2 Is Not Sufficient to Sensitize the Cells to Fas-Mediated Apoptosis

Resistance of melanoma cells to Fas-induced apoptosis has previously been mainly correlated with the expression of antiapoptotic protein Bcl-2 (24), and Bcl-2 is expressed in the melanoma cell lines used here, regardless of their susceptibility to Fas-induced apoptosis. Therefore, we sought to investigate the role of Bcl-2 by examining whether Bcl-2 knockdown would modulate the resistance of melanoma cell lines to Fas-induced apoptosis. Transfection of the resistant melanoma cells with Bcl-2-specific siRNA significantly down-regulated the level of Bcl-2 protein but did not affect spontaneous or Fas-induced apoptosis (Fig. 7). However, simultaneous knockdown of both Bcl-2 and Mcl-1 led to a slight increase in the percentage of apoptotic cells in response to Fas compared with Mcl-1 knockdown alone (Fig. 7). Thus, Mcl-1, rather than Bcl-2, seems to have a predominant role in conferring resistance to Fas-induced apoptosis.

Mcl-1 Expression Is Up-Regulated by the MAPK/ERK Pathway

The MAPK/ERK and phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathways are major pathways implicated in growth and survival of melanoma cells (29-31). To examine the possible regulation of Mcl-1 expression by either pathway,

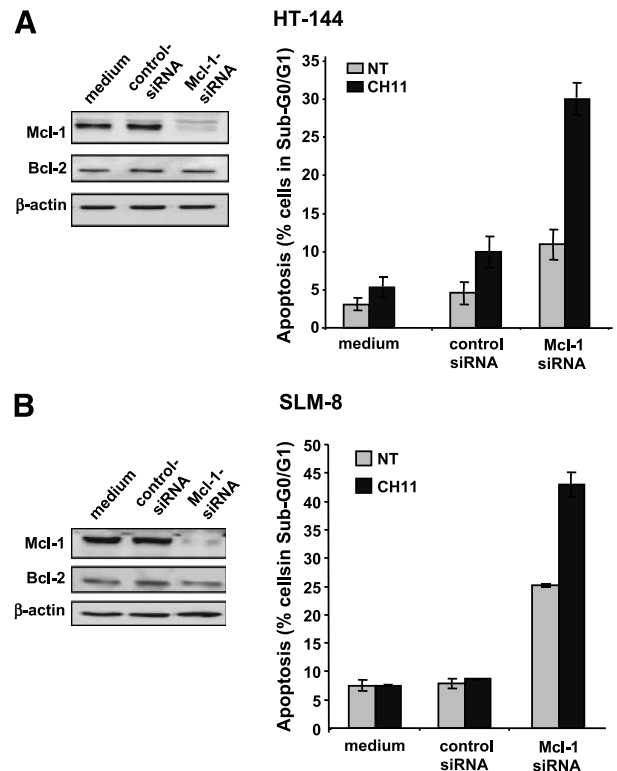


FIGURE 5. Knockdown of Mcl-1 also sensitizes HT-144 (**A**) and SLM-8 (**B**) cells to Fas-induced apoptosis. The cells were transfected with Mcl-1-specific siRNA or negative control siRNA and treated as described in Fig. 4. The expression of Mcl-1 and Bcl-2 proteins was then verified by immunoblot analysis, and apoptosis was determined by nuclear DNA content and FACS analysis. Columns, mean percentages of cells in sub-G₀-G₁ region from three independent experiments done in triplicate; bars, SEs.

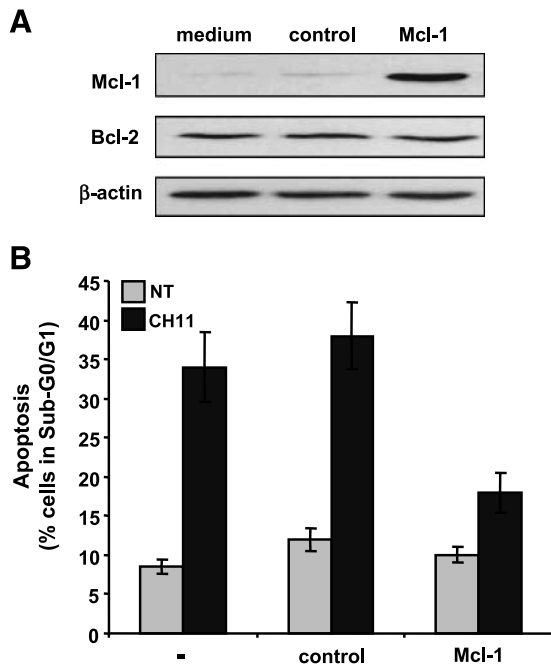


FIGURE 6. Overexpression of Mcl-1 protects M74 cells from Fas-induced apoptosis. The cells were cotransfected or not with Mcl-1 or control plasmids together with the GFP plasmid as described under Materials and Methods. **A.** The expression of Mcl-1 in the GFP-expressing cells was determined by immunoblot analysis. **B.** The GFP-sorted cells were treated with or without anti-Fas mAb CH11 for 24 h, and apoptosis was determined by nuclear DNA content and FACS analysis. The results are representative of three independent experiments.

Mel-1 cells were treated with the MAPK/ERK kinase-1 (MEK-1) inhibitor U0126 or with the PI3K/AKT inhibitor LY294002. Treatment of the cells with the MEK-1 inhibitor decreased the expression of Mcl-1 both at mRNA (Fig. 8A) and protein (Fig. 8B) levels, whereas the levels of β -actin remained unchanged. Mel-1 cells have constitutive active ERK1/2, and their treatment with the MEK-1 inhibitor led to the inhibition of ERK1/2 phosphorylation (Fig. 8C). In contrast, the expression of Mcl-1 is not affected by the PI3K/AKT inhibitor, despite the fact that it inhibits the activation of AKT, which is also constitutive in Mel-1 cells (Fig. 8D). Similar results were also obtained with HT-144 melanoma cells (data not shown).

Because Mcl-1 is important in Fas-mediated apoptosis and is regulated by the MAPK/ERK pathway, we then verified if inhibition of the ERK1/2 activation would sensitize the cells to Fas-induced apoptosis. Mel-1 and HT-144 cells were pretreated with U0126 for 4 h and then triggered with anti-Fas mAb CH11. Treatment with U0126 alone induced a 20% of apoptosis, and combination of U0126 with CH11 increased apoptosis by 2-fold up to 40% to 45% (Fig. 9).

Because treatment of the cells with the U0126 led to a significant apoptosis after 24 h, we verified that the down-regulation of Mcl-1 is not a consequence of cell death. The results shown in Fig. 10A and B indicate that the down-regulation of Mcl-1 mRNA and protein levels after treatment with U0126 inhibitor occurs after 2 to 4 h of treatment, a time period that precedes the commitment of the cells to apoptosis. Indeed, U0126-induced apoptosis was observed only after 18 to

24 h of treatment (data not shown). Furthermore, we examined the regulation of Mcl-1 expression in apoptotic conditions, i.e., in cells treated with the Mek-1 inhibitor and anti-Fas mAb (24-h period), in the presence or absence of the pan-caspase inhibitor z-VAD. As shown in Fig. 10C, the Mek-1 inhibitor significantly reduces Mcl-1 protein level even in the presence of the caspase inhibitor, which blocks apoptosis by 80% (data not shown). Together, these results suggest that the reduction of Mcl-1 by the Mek-1 inhibitor is not a consequence of cell death, but rather, the MAPK/ERK pathway is a major signaling pathway involved in the regulation of Mcl-1 expression in malignant melanoma.

To further confirm the effects of the Mek-1 inhibitor on both Mcl-1 expression and apoptosis, we have assessed the effects of a dominant-negative form of Mek-1 (DN-Mek-1). Transfection of the cells with a DN-Mek-1 plasmid led to a significant reduction of Mcl-1 protein level compared with nontransfected cells and to cells transfected with a control plasmid (Fig. 11A). Furthermore, expression of the DN-Mek-1 also sensitized the cells to Fas-induced apoptosis (Fig. 11B). Altogether, these

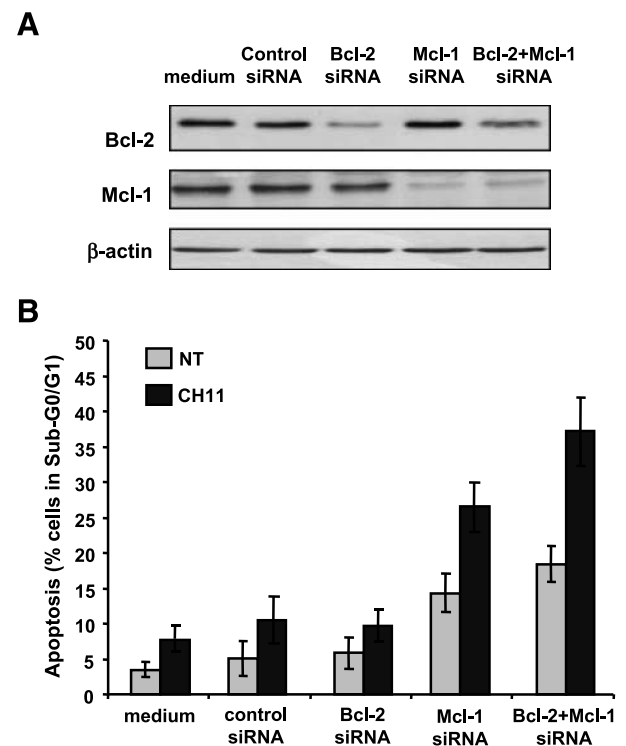


FIGURE 7. Knockdown of Bcl-2 does not sensitize melanoma cells to Fas-induced apoptosis. **A.** Bcl-2-specific siRNA, negative control siRNA, Mcl-1-specific siRNA, or both Bcl-2-specific and Mcl-1-specific siRNA were introduced into Mel-1 cells using Oligofectamine transfection reagent. The cells were harvested after 48 h, and whole-cell lysates were prepared and subjected to immunoblot analysis using Bcl-2, Mcl-1, and β -actin-specific antibodies. The results are representative of three independent experiments. **B.** Mel-1 cells were transfected or not (medium) with control siRNA, Bcl-2-specific siRNA, Mcl-1-specific siRNA, or both Bcl-2-specific and Mcl-1-specific siRNA. At 48 h after transfection, the cells were treated or not with anti-Fas mAb CH11 (500 ng/mL, 24 h), and their apoptosis was determined by nuclear DNA content and FACS analysis. Columns, mean percentages of cells in sub-G₀-G₁ region from three independent experiments done in triplicate; bars, SEs. Similar results were obtained with other Fas-resistant melanoma cell lines (data not shown).

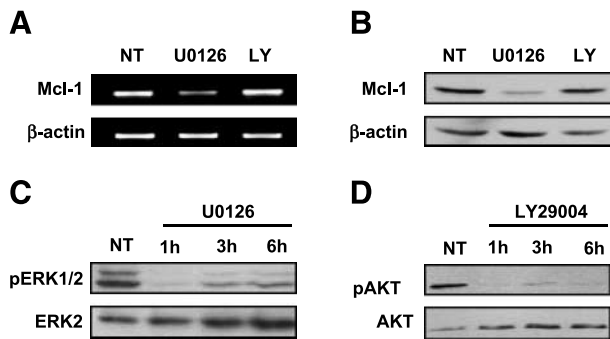


FIGURE 8. MAPK/ERK regulates the expression of Mcl-1. Mel-1 cells were treated or not with the MEK-1 inhibitor U0126 (10 μ mol/L) or with the PI3K/AKT inhibitor LY294002 (25 μ mol/L) for 12 h, and then the expression of Mcl-1 was determined by reverse transcription-PCR (A) or by immunoblot analysis (B). U0126 (C) and LY294002 (D) suppress activation of ERK1/2 and AKT, respectively. Cells were treated with the inhibitors for the indicated periods of time, and activation of ERK1/2 and AKT was determined by immunoblot analysis using specific antibodies that recognize the phosphorylated forms of ERK1/2 and AKT. The results are representative of three independent experiments.

results indicate that, in malignant melanoma cells, resistance to Fas-mediated apoptosis is conferred, at least in part, by the MAPK/ERK/Mcl-1 signaling pathway.

MAPK/ERK Does Not Regulate Mcl-1 through Protein Stabilization

Our results show that MAPK/ERK regulates both mRNA and protein levels of Mcl-1. Because Mcl-1 protein is known to have a short half-life and to be regulated by protein degradation through the proteasome pathway (32-34), we have examined if inhibition of the MAPK/ERK signaling pathway reduces these levels by increasing protein turnover through the proteasome pathway. Accordingly, treatment of the cells with the proteasome inhibitor MG132 for 1 h led to a significant increase in Mcl-1 protein level, which remains elevated after 4 h of treatment (data not shown). Addition of the Mek-1

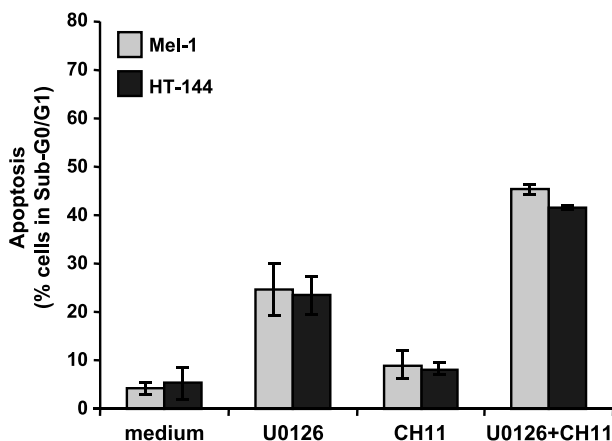


FIGURE 9. The MEK-1 inhibitor U0126 sensitizes melanoma cell lines to Fas-induced apoptosis. Mel-1 and HT-144 cells were preincubated or not with 10 μ mol/L of U0126 for 4 h and then treated with anti-Fas mAb CH11 (500 ng/mL). Apoptosis was determined 24 h later by determining nuclear DNA content and FACS analysis. Columns, mean percentages of cells in the sub-G₀-G₁ region from triplicates and representative of three independent experiments; bars, SEs.

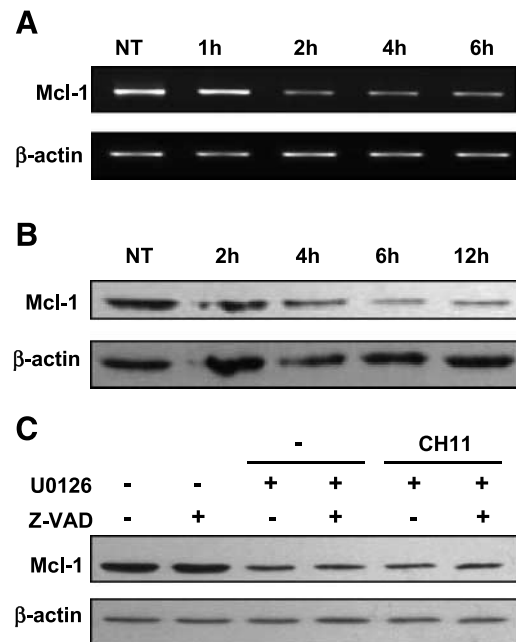


FIGURE 10. The MAPK/ERK controls the expression of Mcl-1 independently from cell apoptosis. The cells were treated with the Mek-1 inhibitor U0126 for different periods of time, and the expression of Mcl-1 was then determined by reverse transcription-PCR (A) and immunoblot analysis (B). In C, the cells were treated as indicated with the U0126 and anti-Fas mAb CH11 for 24 h in the presence or absence of 100 μ mol/L of the pan-caspase inhibitor z-VAD. The expression of Mcl-1 was determined by immunoblot analysis. The results are representative of three independent experiments.

inhibitor to the MG132-treated cells was still able to reduce the levels of Mcl-1 protein (Fig. 12). This suggests that the MAPK/ERK pathway does not regulate Mcl-1 through protein stabilization/proteasome but rather regulates the mRNA level as shown above (Figs. 8 and 10).

Discussion

Resistance of malignant/metastatic melanoma cells to death receptor-mediated apoptosis is now recognized as an important mechanism by which these tumors evade the immune system. In this study, we have shown that Mcl-1 protein, a member of the Bcl-2 antiapoptotic family, plays a major role in the resistance of melanoma cells toward Fas-mediated apoptosis. Several studies have shown that melanoma tumor cells express variable levels of Fas *in vivo* and *in vitro* but were found to be resistant to Fas-induced apoptosis (35, 36), suggesting that additional mechanisms at the level of Fas-induced caspase activation are likely to contribute to the observed resistance. We show that Mcl-1 is constitutively expressed in resistant melanoma cell lines and its knockdown using siRNA sensitizes these cells toward Fas-induced apoptosis, whereas overexpression of Mcl-1 protects sensitive melanoma cell lines from Fas-induced apoptosis. We further show that the expression of Mcl-1 is regulated by the MAPK/ERK signaling pathway, a crucial survival pathway in melanoma cells, most likely through the regulation of its mRNA level. Thus, our study provides the first evidence on the implication of MAPK/ERK/Mcl-1 signaling pathway in the resistance of melanoma cells to Fas-mediated apoptosis.

Most melanoma cells were shown to express Bcl-2, Bcl-xL, and Mcl-1 (37, 38). Additional studies have correlated the resistance of melanoma cells to Fas-mediated apoptosis, mainly with the expression levels of proapoptotic Bax and antiapoptotic Bcl-2 proteins (24-26), whereas expression of Bcl-xL has been largely associated with their drug resistance (39-41). The melanoma cell lines used in our study express Bcl-2 and Bcl-xL; however, only Mcl-1 expression was decreased after cycloheximide treatment. The decrease in Mcl-1 protein levels is rapid and occurs before the onset of Fas-induced apoptosis, which is consistent with previous studies, demonstrating that Mcl-1 protein has a rapid turnover with a half-life of 30 min to 3 h (42-44). Along these lines, knockdown of Mcl-1 alone, but not Bcl-2, was sufficient to sensitize melanoma cells to Fas-induced apoptosis, and overexpression of Mcl-1 significantly reduced Fas-induced apoptosis of Fas-sensitive Bcl-2-expressing M74 melanoma cells. Therefore, our study attributes to the antiapoptotic Mcl-1 protein a major role in controlling the resistance of melanoma cells to Fas-mediated apoptosis. However, Bcl-2 might have a secondary role in this process, because simultaneous knockdown of Mcl-1 and Bcl-2 led to more apoptotic cells in response to Fas triggering than Mcl-1 knockdown alone. These results are consistent with the fact that, in primary and metastatic melanomas, Mcl-1 levels are higher than Bcl-2; the levels of which are mainly associated with melanocytes and benign nevi (38, 45-47). Our studies

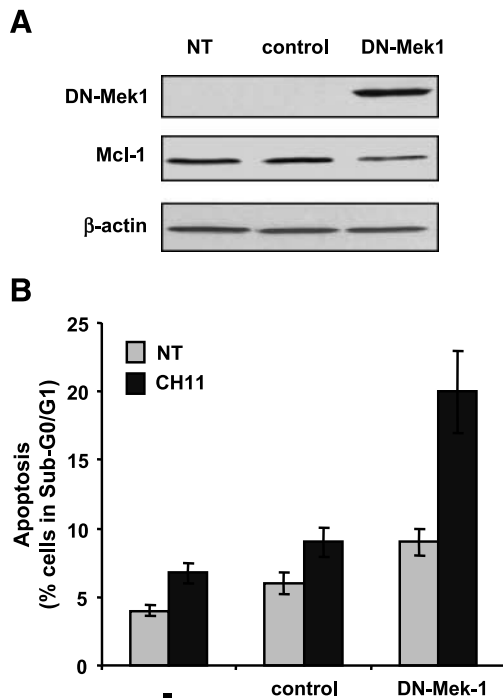


FIGURE 11. Dominant-negative form of Mek-1 (DN-Mek-1) reduces Mcl-1 and sensitizes melanoma cells to Fas-induced apoptosis. Mel-1 cells were cotransfected or not with DN-Mek-1 or control plasmid together with GFP plasmid as described under Materials and Methods. **A.** Expression of DN-Mek-1 and Mcl-1 proteins in GFP-expressing cells was determined by immunoblot analysis. **B.** The GFP-sorted cells were treated with or without anti-Fas mAb CH11 for 24 h, and apoptosis was determined by nuclear DNA content and FACS analysis. The results are representative of three independent experiments.

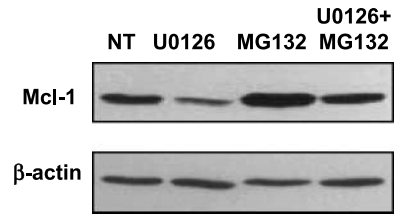


FIGURE 12. The MAPK/ERK regulates the expression of Mcl-1 independently from the proteasome pathway. The cells were pretreated or not with the MG132 (10 μ mol/L) for 1 h and then treated or not with the Mek-1 inhibitor U0126 for 3 h. Expression of Mcl-1 was then determined by immunoblot analysis. The results are representative of three independent experiments.

might suggest a functional hierarchy of the antiapoptotic Bcl-2 proteins in melanoma cells resistant to Fas-induced apoptosis; Mcl-1 is required, as well as, to a significantly lesser extent, Bcl-2 and perhaps Bcl-xL. This is in line with recent evidence about the function of Bcl-2-related proteins in resistance of melanoma cells to bortezomib-induced death (48). Beside, the implication of additional antiapoptotic proteins in the resistance of melanoma cells to Fas-mediated apoptosis is not excluded. Recently, XIAP, a member of the IAP family of proteins known to inhibit both activation of caspase-9 and caspase-3, has been implicated in the resistance of melanoma cells to TRAIL-induced apoptosis (49). Although XIAP is expressed in our melanoma cell lines, it does not seem to be implicated in their cycloheximide sensitization for Fas-mediated apoptosis, because treatment with cycloheximide had no effect on its levels (data not shown). Furthermore, we found that Mel-1 cells express weak levels of c-Flip, an endogenous inhibitor of caspase-8 activation, and cycloheximide did not modulate the levels of c-Flip (data not shown).

The role of Bcl-2 and Bcl-xL in the regulation of death receptor-induced and drug-induced apoptosis has been established in various cell types (28, 50, 51). A predominant role of Mcl-1 in the regulation of Fas apoptosis has recently been described in leukemia cells (52, 53) and in human hepatocytes (54). Our study identifies melanoma cells as an additional lineage, wherein Mcl-1 regulates Fas-mediated apoptosis and seems to have a predominant role.

The mechanisms by which Mcl-1 inhibits Fas-induced apoptosis are still unclear. Mcl-1 is known to block apoptosis at the mitochondria level by interacting and inhibiting the action of Bcl-2 proapoptotic proteins (55). Melanoma cells are type II cells, and in these cells, the weak activation of caspase-8 at the death-inducing signaling complex activates the mitochondria death pathway and subsequently caspase-3, which in turn leads to the processing of the large majority of caspase-8 and to the amplification of the weak initiating signal generated at the death-inducing signaling complex. Therefore, it is likely that Mcl-1 blocks Fas-induced apoptosis downstream of caspase-8 activation by affecting the mitochondria and caspase-3 activation, thereby inhibiting the amplification loop necessary for the execution of apoptosis in type II cells. This could explain the results in Fig. 2B, wherein the cleavage of caspase-8, in response to Fas engagement, is seen only in the presence of cycloheximide, which inhibits the expression of Mcl-1. Along these lines, our results show that the

knockdown of Mcl-1 (Fig. 4C) and cycloheximide (Fig. 2B) lead to the activation of caspase-9 downstream of Fas, which is necessary for the activation of caspase-3 and the subsequent amplification loop.

The inhibition of mitochondrial death pathway by Mcl-1 could proceed through several mechanisms. In this context, it was recently shown that Mcl-1 can interact with truncated bid (truncated Bid) and inhibits its ability to activate the mitochondrial death pathway downstream of death receptors (56). Because melanoma cells are type II cells, they are likely to depend on the activation of Bid (Fig. 2C) to activate the mitochondria death pathway, suggesting that Mcl-1 can inhibit Fas-induced apoptosis in melanoma cells by interacting with truncated Bid, thereby preventing it from activating Bax and/or Bak. However, Mcl-1 can also inhibit the mitochondria death pathway by interacting with additional proapoptotic proteins, such as Bak and Bim (57-59). Thus, to reach an understanding into how Mcl-1 confers Fas resistance in melanoma cells, additional experiments are needed to determine if Bim and Bak or other Bcl-2 proapoptotic proteins are implicated in Fas-induced apoptosis and to identify the Mcl-1-interacting partner(s), such as truncated Bid, Bak, Bim, or others, in these cells.

Our results also show that the knockdown of Mcl-1 increased the spontaneous apoptosis of melanoma cells. This effect has also been observed in recent studies using different cellular models (57, 60). Because melanoma cells are known to produce Fas ligand as one of their counterattack mechanisms (61, 62), it is conceivable that the observed spontaneous apoptosis after the knockdown of Mcl-1 could be mediated by the autocrine activation of the Fas-Fas ligand system. Additional experiments are warranted to understand how Mcl-1 controls spontaneous apoptosis in melanoma cells.

Using the Mek-1 inhibitor and a dominant-negative form of Mek-1, we showed that expression of Mcl-1 is regulated by the MAPK/ERK signaling pathway in melanoma cells. Previous studies have reported that Mcl-1 is regulated by the PI3K/AKT signaling pathway in human lung and hepatocyte carcinoma cells (54, 63, 64). In the hematopoietic cell line TF-1, MAPK/ERK was suggested to control Mcl-1 transcription, whereas the PI3K/AKT controls Mcl-1 translation (65). In our cell model, inhibition of the PI3K/AKT did not reduce either transcription or translation of Mcl-1, thus excluding its implication. A previous study has shown that in melanoma cells, Mcl-1 is regulated by the Src/Stat-3 signaling pathway, which also controls spontaneous apoptosis (66). Although our results indicate that the MAPK/ERK is involved in the regulation of Mcl-1, we do not exclude the implication of additional signaling pathways, such as Src/Stat-3. Whether there is a cross-talk between these signaling pathways in melanoma cells is unknown and deserves to be investigated. In agreement with our findings is a recent report indicating that, in prostate cancer cells, Mcl-1 is regulated by the MAPK/ERK pathway (67).

The mechanisms by which the MAPK/ERK regulates the expression of Mcl-1 are still unclear. Our results show that inhibition of the MAPK/ERK inhibits the levels of Mcl-1 mRNA. Mcl-1 protein is tightly regulated by the proteasome pathway (32-34) and has been shown to be phosphorylated by ERK, resulting in protein stabilization and protection from

proteasome degradation (68). However, this is unlikely to be the case in our melanoma cell lines, as Mcl-1 protein levels were still inhibited by the Mek-1 inhibitor even in the presence of the proteasome inhibitor. Accordingly, the MAPK/ERK pathway is most likely regulating Mcl-1 expression at the mRNA level in melanoma cells, as it has been suggested in TF1 cells (65). Whether the MAPK/ERK regulates Mcl-1 protein translation remains, however, to be investigated. Studies aimed at understanding the regulation of Mcl-1 expression by the MAPK/ERK pathway are currently under way.

We also found that inhibition of the MAPK/ERK pathway sensitizes resistant melanoma cell lines to Fas-induced apoptosis. We, thus, propose that the MAPK/ERK pathway promotes Fas resistance in part by up-regulating the level of Mcl-1. Our results do not exclude that MAPK/ERK pathway activates additional survival mechanisms likely to be important in the resistance to Fas-induced apoptosis. We have recently shown that activation of ERK inhibits Fas-induced apoptosis in the Fas-sensitive A375 melanoma cell line by reducing activation of caspase-8 (69). Activated ERK can also reduce the release of the apoptogenic factor Smac/DIABLO from the mitochondria, allowing the activation of IAP proteins and, thereby, inhibition of executioner caspases, an event implicated in the resistance to TRAIL-induced apoptosis (31).

Mcl-1 is highly expressed in hematopoietic cells and in leukemia tumors (70-73). In addition to our study, Mcl-1 has recently been shown to be involved both in the chemosensitization or radiosensitization of melanoma tumors (74-76), and in resistance of other solid tumors to TRAIL-induced apoptosis (77). In melanoma, resistance to TRAIL has been rather attributed to other antiapoptotic proteins, such as c-Flip and XIAP (31, 49, 78). Although not addressed in this study, loss of Mcl-1 may also partially contribute to sensitization of these tumors to TRAIL because TRAIL-induced apoptosis in melanoma cells is largely dependent on activation of mitochondrial death pathway (79). Moreover, clinical studies have indicated that overexpression of Mcl-1 is correlated with advanced clinical stages of melanoma (38, 80). Interestingly, resistance of melanoma cells to Fas-mediated apoptosis also correlates with advanced stages of melanoma (5, 81, 82). Thus, our study points to the importance of Mcl-1 in the resistance of advanced melanomas to Fas-induced apoptosis and reinforces the notion of Mcl-1 as a putative therapeutic target in these tumors.

Materials and Methods

Cell Lines, Antibodies, and Reagents

The Mel-1 and HT-144 melanoma cell lines used in this study were kindly provided by Dr. E. Tartour (Institut National de la Sante et de la Recherche Medicale U255, Paris, France) and the melanoma cell line SLM8 by Dr. M. Viguier (Hopital Saint Louis, Paris, France). The cells were grown in RPMI 1640 supplemented with 10% FCS, 2 mmol/L glutamine, and 100 units/mL penicillin and streptomycin. Cycloheximide, the Mek-1 inhibitor U0126, and the PI3K/Akt inhibitor LY294002 were purchased from Calbiochem. The proteasome inhibitor MG132 was from Sigma. The antibodies used in this study were obtained as follows: caspase-8, caspase-9, Bcl-xL, phosphorylated AKT (Ser⁴⁷³), and

AKT were from Cell Signaling Technologies; caspase-3, Mcl-1, Bcl-2, and phosphorylated p44/42 MAPK (E-4), which recognizes the active forms of ERK1/2, ERK2 (C-14), Bid (FL-195 and 5C9), and β -actin (C-2) were from Santa Cruz Biotechnology; and the mouse anti-human Fas monoclonal antibodies (CH11 and UB2) were purchased from Kamiya Biomedicals.

Cell Death and Determination of Apoptosis

Melanoma cells were resuspended at 1×10^6 /mL in complete RPMI medium and then seeded in 24-well plates (1×10^5 per well) for an overnight culture. The cells were then washed with PBS and cultured in RPMI containing 2.5% FCS alone or in the presence of inhibitors (cycloheximide, U0126, or LY294002), after which they were treated with the anti-Fas mAb CH11. At the end of cultures, apoptosis was determined by nuclear DNA fragmentation by staining the cells with propidium iodide and flow cytometry analysis using a FACScan (BD Biosciences). Briefly, after stimulation, the cells were pelleted, resuspended in 300 μ L cold PBS, and fixed on ice for 30 min in ethanol (70%). The cells were then washed and incubated in PBS containing propidium iodide (25 μ g/mL) and RNase A (10 μ g/mL) for 30 min at room temperature. Detection of DNA fragmentation in nuclei of apoptotic cells was done using cell cycle analysis and flow cytometry, wherein cells with DNA content lower than 2N appear in the sub-G₀-G1 region. Cell apoptosis was also determined by the Annexin V-PE/7-AAD detection kit from BD PharMingen. After stimulation, the cells were washed in cold PBS and incubated in fluorescence-activated cell sorting (FACS) buffer containing 5 μ L of Annexin V-PE and 5 μ L of 7-AAD for 15 min at room temperature in the dark. The cells were then washed and analyzed by flow cytometry using FACScan.

Caspase Activities, ERK and AKT Phosphorylation, and Immunoblot Analysis

At the end of cultures, melanoma cells were detached and washed with cold PBS and lysed in radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors as we previously described (83). Activation of caspase-8, caspase-9, and caspase-3 was determined by immunoblot analysis using specific antibodies that recognize both the inactive procaspase forms and the cleaved active caspase forms (p43/41 and p18 for caspase-8, p35 for caspase-9, and p19 and p17 for caspase-3). ERK1/2 and AKT activation were determined by immunoblot analysis using anti-phosphorylated ERK1/2 (E-4) and anti-phosphorylated AKT antibodies, respectively. The expression levels of Bid, Mcl-1, Bcl-xL, and Bcl-2 were detected by blotting with specific antibodies. Immunoblots were stripped and reprobed with control antibodies to ensure equal loading. In all experiments, immunoblots were visualized using an horseradish peroxidase-conjugated secondary antibody followed by enhanced chemiluminescence's detection (Pierce).

RNA Isolation and Reverse Transcription-PCR

Total RNA was extracted with Trizol reagent according to the manufacturer's instructions (Life Technologies). First-strand cDNA was prepared from 1 μ g of total RNA using the

Thermoscript reverse transcription-PCR system from Invitrogen. Mcl-1 and β -actin transcripts were amplified by PCR using specific primers; Mcl-1 forward 5'-CGG-CGA-TCG-CTG-GAG-ATT-AT-3', Mcl-1 reverse 5'-GTG-GTG-GTT-GGT-TA-3', β -actin sense 5'-AGC-CAT-GCC-AAT-CTC-ATC-TTG-T-3', β -actin antisense 5'-AGC-GCT-GCT-TCC-AGC-TCC-TC-3' (53, 84). PCR reactions were carried out with 1 unit of Taq polymerase in a total volume of 50 μ L, and amplifications were carried out in a Peltier Thermal Cycler from MJ Research. The amplification of each gene was in the linear curve. Mcl-1 amplification was done for 30 cycles (94°C for 30 s, 58°C for 1 min, and 72°C for 1 min) and β -actin amplification for 20 cycles (94°C for 35 s, 58°C for 35 s, and 72°C for 40 s). An additional 10 min of extension at 72°C was done for each gene amplification. PCR products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining.

RNA Interference

Silencer Validated siRNA specific for Mcl-1 (siRNA ID 120644 sense 5'GGACUUUUUAUACCGUUUAUtt3', antisense 5'AUAACAGGUAAUAAAAGUCctg3'), Silencer Predesigned siRNA specific for Bcl-2 (siRNA ID 42815 sense 5'GGAUUGUGGCCUUCUUUGAtt3', antisense 5'UCAAAAGAA-GGCCACAAUCCt3'), and Silencer Negative Control siRNA were purchased from Ambion. One day before siRNA transfection, cells were seeded at a density of 1×10^5 per well of six-well plate to achieve roughly 40% to 50% confluence. Just before transfection, growth media of the cells was replaced with 800 μ L of OptiMEM media (Life Technologies) without FCS. Cells in OptiMEM media were transfected with 200 nmol/L Mcl-1-specific or Bcl-2-specific siRNA or Negative Control siRNA using Oligofectamine reagent (Invitrogen) according to manufacturer's transfection protocols and to ref. (85). After 5 h, 1 mL of growth media was added to the transfection media, and 24 h later, the media was completely replaced by growth media. Inhibition of Mcl-1 protein expression and Fas-mediated apoptosis were assessed after 48 h of transfection by immunoblot analysis and by nuclear DNA fragmentation, respectively, as described above.

Plasmids and Cell Transfection

The flag-tagged Mek-1 dominant-negative and Mcl-1 plasmids were generous gifts of Dr. Jean Charron (Laval University, Canada) and Dr. Shaomeng Wang (University of Michigan, MI) respectively. The GFP plasmid was a generous gift of Dr. Kristiina Vuori (Burnham Institute, La Jolla, CA). Melanoma cells were transfected using effecten (Qiagen) as we previously described (69). Cells were plated at 50% to 60% of confluence and were cotransfected with a total of 5 μ g of DNA with either control plasmid, DN-Mek-1 plasmid, or Mcl-1 plasmid together with GFP plasmid at 5:1 ratio. The cells were incubated with effecten-DNA complexes for 12 h and washed with PBS, and fresh growth medium was added. At 24 h later, the cells were washed with PBS and GFP-expressing cells were sorted by FACS analysis and used in subsequent experiments. Expression of DN-Mek-1 and Mcl-1 was verified after transfection by immunoblot analysis using anti-flag and anti-Mcl-1 mAbs, respectively.

Fas Expression and Flow Cytometry

Expression of Fas receptor on melanoma cells was determined by staining the cells with FITC-conjugated anti-Fas mAb (UB2) and FACS analysis as we previously described (69).

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