Expression of Apoptosis Regulators in Cutaneous Malignant Melanoma

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ABSTRACT

Metastatic malignant melanoma (MM) is usually incurable and responds poorly to chemotherapy. Because many cytotoxic drugs cause cell death by inducing apoptosis, an imbalance of apoptosis regulatory proteins may contribute to MM treatment resistance. We have previously shown reduced expression of Bcl-2 protein, a negative regulator of apoptosis, in MM as compared with benign nevi. It is hypothesized that other apoptosis regulators may be involved in survival of MM cells. We examined the expression of Bax, Bcl-2, Bcl-X, and Mcl-1 in human benign nevi, primary MM, and metastatic MM using immunohistochemistry. Results were confirmed with Western blotting. The proapoptotic protein, Bax, was surprisingly overexpressed in all MM samples compared with benign nevi. Interestingly, in most MM samples there was overexpression of Mcl-1 or Bcl-XL, both negative regulators of apoptosis. Increased expression of Mcl-1 and Bcl-XL was first observed in thin primary melanomas, suggesting that up-regulation of these proteins represents a relatively early event associated with malignant transformation in MM. As published previously, the majority of primary and metastatic MM exhibited reduced Bcl-2 levels. We conclude that the apoptosis inhibitors Bcl-XL or Mcl-1, alone or in combination, may circumvent the normal cell death pathway, contributing to the pathogenesis and treatment resistance in metastatic MM.

INTRODUCTION

Metastatic MM is usually incurable, with a 5-year survival <10% and a poor response to chemotherapy (1, 2). Because many cytotoxic drugs cause cell death by inducing apoptosis (programmed cell death) (3-5), an imbalance of apoptosis regulatory proteins may contribute to MM treatment resistance. Apoptosis in MM has not been examined extensively, but two studies have demonstrated low levels of spontaneous apoptosis in human MM (6, 7), and MM cell lines have been shown to be extremely resistant to apoptotic cell death after hyperthermia (8).

Apoptosis is regulated in part by Bcl-2 and its related proteins (9-12). Many human cancers overexpress Bcl-2, an inhibitor of apoptosis, thus potentially contributing to neoplastic expansion and resistance to drug-induced tumor cell killing (13). Elevated levels of Bcl-2 protein markedly increase the drug resistance of tumor cell lines (10, 13) and are associated with poor prognosis in some solid tumor types, such as prostate carcinoma (14, 15). There is, however, no correlation between Bcl-2 overexpression and adverse outcome in other malignancies such as breast (16-20), ovarian (21, 22), colon (23), lung (24), and thyroid carcinomas (25). In human metastatic MM, Bcl-2 protein is decreased relative to benign nevi and normal melanocytes (26-29), and Bcl-2 expression does not correlate with clinical outcome in uveal MM (30). These data suggest that treatment resistance in MM may be mediated largely by apoptotic inhibitors other than Bcl-2.

Bcl-2 is a member of a multigene family. Some Bcl-2 family proteins function as inhibitors of apoptosis (Bcl-2, Mcl-1, and Bcl-XL), whereas others promote cell death (Bax and Bcl-XS). Interactions among proteins of this family, with formation of both homo- and heterodimers, regulate the sensitivity of cells to apoptotic stimuli (31). Homodimers of Bax, a Mr 21,000 protein with sequence homology to Bcl-2, actively induce or promote cell death, and the ratio of Bax to apoptosis inhibitors such as Bcl-2 determines the degree of heterodimer formation and sensitivity of the cell to apoptosis (32). Mcl-1, another Bcl-2 homologue, has been shown to delay c-Myc-inducible apoptosis in Chinese hamster ovary cells and functions, like Bcl-2, to neutralize Bax-mediated cell death in a yeast system (31, 33, 34). Bcl-X is another important apoptosis regulator: alternative splicing results in both a long (Bcl-XL) and short form (Bcl-XS) of this protein (35). Bcl-XL blocks cell death, but Bcl-XS promotes apoptosis.

Because there is no evidence that metastatic MM overexpresses Bcl-2, we hypothesized that reduced levels of the proapoptotic protein Bax, or increased expression of other apoptosis inhibitors such as Mcl-1 or Bcl-XL, might be important in preventing programmed cell death and drug-induced apoptosis in this therapy-resistant tumor. Variable Bax mRNA levels have

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3 The abbreviation used is: MM, malignant melanoma.
been demonstrated in metastatic MM by reverse transcription-PCR (36), but expression of Bax, Mcl-1, and Bcl-X proteins in MM has not been examined previously. In this study, we analyzed the protein levels of Bax, Bcl-2, Bcl-X, and Mcl-1 in cutaneous MM using immunohistochemistry and Western blotting analysis in human tumor samples.

MATERIALS AND METHODS

Human Melanoma Specimens. For immunoblotting experiments, 10 tissue samples of human metastatic MM were acquired from nine patients. Three of the metastases were from patients that had received prior chemotherapy. However, in one patient, adjuvant chemotherapy was given 8 years previous for an unrelated malignancy. A 1-cm³ portion of tumor was removed from the metastatic mass and instantly frozen in liquid nitrogen. At least six additional tissue blocks were submitted for pathological analysis to confirm the diagnosis and evaluate surgical resection margins. Control samples of common acquired nevi were obtained from freshly resected lesions that were clinically completely benign with no suspicious features. Patient consent was obtained in all cases. A small fragment of nevus (approximately one-fourth of the specimen) was snap frozen in liquid nitrogen, and the balance of the tissue was submitted in its entirety for histopathological diagnosis. Protein was not extracted from frozen tissue until the clinical impression of benign common acquired nevus was confirmed histopathologically by a dermatopathologist (M. J. T. or V. A. T.).

For the immunohistochemistry analysis, formalin-fixed, paraffin-embedded tissue was randomly obtained from the files of Vancouver Hospital. Five common acquired nevi, 10 primary cutaneous melanomas, and 5 metastatic melanomas were selected randomly.

Immunoblot Analysis. Frozen tissue samples were rapidly thawed and homogenized with a Polytron in RIPA buffer (1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS). Protein concentration was measured with a Protein Assay kit (Bio-Rad, Hercules, CA), and a total of 100 µg of protein per sample was separated on a 12% polyacrylamide gel, transferred to nitrocellulose membrane, and probed with primary antibodies against Bax (rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-X (rabbit polyclonal; Santa Cruz Biotechnology), Mcl-1 (rabbit polyclonal; Santa Cruz Biotechnology), and Bcl-2 (mouse monoclonal; Dako, Carpinteria, CA). All primary antibodies were used at a dilution of 1:300. Binding of the primary antibody was detected using horseradish peroxidase-conjugated goat anti-rabbit (BRL, Mississauga, Ontario, Canada) or goat anti-mouse (Santa Cruz Biotechnology) secondary antibody. Signals were detected using enhanced chemiluminescence detection (ECL; Amersham, Arlington Heights, IL). The same membrane was stripped and reprobed successively with different primary antibodies. Western blotting was performed at least twice for each tissue sample, and similar results were obtained.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue from common acquired nevi and primary and metastatic MM specimens were used for immunohistochemistry. Immunohistochemical staining was performed using a standard streptavidin-biotin technique after microwave antigen retrieval. Slides were then incubated with primary antibodies against Bcl-X (37), Mcl-1 (38), Bax (39), and Bcl-2 (28) as described previously. A biotinylated goat anti-rabbit secondary antibody was used for Bcl-X, Mcl-1, and Bax, whereas a biotinylated goat anti-mouse secondary antibody was used with Bcl-2. This was then followed by incubation with peroxidase-labeled streptavidin. 3-Amino-9-ethylcarbazole (AEC) substrate was used as the chromagen, and sections were then lightly counterstained with hematoxylin. Appropriate internal controls were used for Bcl-2 (28), Bcl-X (37), Mcl-1 (38), and Bax (39). Antibody specificity was ensured using peptide blocking. Staining intensity was estimated relative to known positive internal controls and scored as 0 (absent), 1 (weak), 2 (moderate), or 3 (strong).

RESULTS

Immunohistochemical Analysis of Apoptosis Regulatory Protein Expression in Primary and Metastatic Melanomas. Immunohistochemistry was used to examine the expression of Bax, Bcl-2, Bcl-X, and Mcl-1 in human MM. Staining was performed on tissue blocks from benign nevi, primary MM, and metastatic MM and summarized in Fig. 1. All MM tumors expressed very strong (3+), diffuse, cytoplasmic staining for Bax protein (Figs. 1A and 2, A–C). All melanomas (Figs. 2, B and C) were found to be greater in immunostaining intensity than benign nevi (Fig. 2A). Bcl-X was also overexpressed in the majority of primary and metastatic MM based on comparisons with benign nevi (Figs. 1B and 2, D–F). Increased cytoplasmic Bcl-X staining was detected in melanoma (Fig. 2F), first noted in shallow primary MM (Fig. 2E). Because this anti-Bcl-X antibody detects both the long and short isoform (37), we immunostained these cases with an antibody with relative specificity for Bcl-Xs. This additional analysis failed to show up-regulated Bcl-Xs expression in MM (data not shown). The antiapoptotic protein, Mcl-1, was more strongly expressed in virtually all MM cases compared with benign nevi melanocytes (Figs. 1C and 2, G–I). Immunostaining was barely detectable in nevi (Fig. 2G), whereas Mcl-1 staining in MM varied from moderate to strong (Fig. 2, H and I). As with Bcl-X, increased Mcl-1 staining was detected initially in melanomas less than 1 mm in thickness (Fig. 2H). In contrast, Bcl-2 expression was decreased in all MM lesions compared with benign nevi (Fig. 1D), consistent with previous studies (26–29).

Immunoblot Analysis of Bax, Bcl-2, Bcl-X, and Mcl-1 in Metastatic Melanoma. To determine the relative expression of the apoptosis regulatory proteins Bax, Bcl-2, Bcl-X, and Mcl-1 in MM, proteins extracted from freshly obtained human metastatic tumors were examined by immunoblotting (Fig. 3). Bax protein was strongly expressed in all MM samples at levels 2–4-fold those found in benign melanocytic nevi as measured by densitometry analysis (data not shown). Of note, a Mr 18,000 Bax band was detected in protein samples derived from nevi but was absent or only weakly present in MM samples. Increased expression of this Mr 18,000 Bax protein has been demonstrated previously in drug-sensitive B-cell chronic lymphocytic leukem-
cells acquire the capacity to avoid drug-induced cell death, defects in regulation of this apoptosis pathway can render a tumor cell more resistant to treatment (3–5). Because metastatic MM responds poorly to chemotherapy (1, 2) and has a low spontaneous rate of apoptosis (6, 7), we hypothesized that MM cells acquire the capacity to avoid drug-induced cell death, possibly by altering levels of apoptosis regulatory proteins. To test this thesis, protein levels of Bax, Bcl-2, Bcl-X, and Mcl-1 were examined in benign melanocytic nevi and MM, using Western blotting and immunohistochemistry. We believe that determination of protein expression in actual human tumor tissue, not just cell lines in culture, is very important in establishing a role for abnormal apoptosis regulation in MM in vivo.

This study demonstrates that the apoptosis inhibitors Bcl-X_L and/or Mcl-1, which are present at very low levels in benign nevi, are overexpressed in MM. Mcl-1 and Bcl-X_L were first observed up-regulated in thin primary melanomas, implying that elevation of these proteins represents an early event in melanoma progression. We confirmed this overexpression using both Western blotting and immunohistochemistry. We believe that overexpression of Bcl-X_L and Mcl-1 supports a role for these proteins in the low rate of apoptosis observed in melanoma (6, 7).

The proapoptotic protein Bax was overexpressed in tissue samples of MM relative to benign nevi. Present models of apoptosis regulation identify Bax as a central activator of the programmed cell death pathway (10). Radiation-induced Bax expression occurs prior to apoptosis of irradiated tumor cells (42, 43), and thus Bax may be important in treatment-mediated tumor cell death. In breast cancer, a marked reduction in Bax immunostaining was observed in 34% of primary tumors from patients who developed metastatic disease: reduced Bax was significantly correlated with treatment failure and shorter survival (44). A relationship between low Bax levels and resistance to apoptosis has also been observed in breast carcinoma (45). Similar to our findings in metastatic MM, strong Bax immunostaining has been noted in thyroid and prostate carcinomas (46, 47). However, in most colorectal adenocarcinomas, Bax immunoreactivity is not significantly altered compared with normal colonic mucosa (48). Mutations that inactivate the Bax gene have been reported in a subset of colonic tumors with microsatellite instability (49).

**DISCUSSION**

It is now recognized that most forms of cancer therapy kill malignant cells by inducing programmed cell death, and that defects in regulation of this apoptosis pathway can render a tumor cell more resistant to treatment (3–5). Because metastatic MM responds poorly to chemotherapy (1, 2) and has a low spontaneous rate of apoptosis (6, 7), we hypothesized that MM cells acquire the capacity to avoid drug-induced cell death, possibly by altering levels of apoptosis regulatory proteins. To
expression in melanoma reported variable Bax mRNA levels using reverse transcription-PCR (36); the investigators did not include benign tissue for comparison, and protein levels were not examined. Our finding of increased Bax protein in MM suggests that this important apoptosis regulator may be nonfunctional, or that elevation of associated antiapoptotic proteins may govern cell death regulation in this tumor. Although Bax is believed to function as a proapoptotic protein, the presence of testicular atrophy in Bax “knock out” mice suggests a cell-protection role for Bax in some tissues (50). Indeed, Bax has been reported to paradoxically promote cell survival in some types of neurons (51). To date, there is
no evidence implicating Bax in protection of MM cells from apoptosis and, in the vast majority of cell types, Bax is proapoptotic.

As an incidental observation, we noted the loss of Mr 18,000 Bax in 6 of 10 metastatic MM samples, although a faint band was detectable in MM cell lines (cell line data not shown). Absence of Mr 18,000 Bax has been associated with resistance to chemotherapy in B-cell chronic lymphocytic leukemia (40), but a definite role for this shorter form of Bax in apoptosis regulation has yet to be determined.

The mechanism underlying Bax elevation in metastatic MM is unknown. Wild-type p53 is known to be an upstream regulator of the Bax gene promoter that contains p53 binding sites and can be directly activated by wild-type but not mutant p53 (43). Most human MM tumors and cell lines lack p53 mutations (52, 53). Overexpression of wild-type p53 is common in metastatic MM and may explain increased Bax expression in vivo. Furthermore, p53 may play a role in regulating MM cell death after chemotherapy, and we have shown recently that melanoma cell lines containing wild-type p53 are sensitive to drug therapy, whereas those containing mutant p53 exhibit treatment resistance (54). In this regard, it is important to note that the studies here have evaluated only basal expression of Bax and other apoptosis regulating proteins. The dynamic alteration of these proteins during genotoxic stress responses was not assessed in this study.

The antiapoptotic protein Mcl-1 was overexpressed in MM compared with benign nevi, as assessed by both Western blotting and immunohistochemistry. Mcl-1 was first identified in a human myeloid leukemia cell line induced to undergo differentiation (33). Mcl-1 heterodimerizes with Bax (31) and can diminish c-Myc-induced apoptosis in Chinese hamster ovary cells (34). In normal tissues, Mcl-1 expression may be differentiation-associated (38), but in both colonic and prostate carcinomas, Mcl-1 immunostaining is the most intense in poorly differentiated, high-grade tumors (47, 48). In MM, a histological indicator of differentiation is melanin pigmentation; in our series of metastatic lesions, there was no correlation of Mcl-1 staining intensity with MM cell pigmentation. Mcl-1 was significantly overexpressed in the majority of MM samples examined, suggesting an important role for this antiapoptotic protein in MM cell survival.

Western blot analysis, using an antibody against amino acids 193–212 of Bcl-X, failed to demonstrate Bcl-X expression in benign melanocytic nevi, but all metastatic MM samples contained a definite Mr ~29,000 band, corresponding to Bcl-XL, the antiapoptotic, long isoform of Bcl-X. Immunohistochemical studies confirmed Bcl-XL overexpression in MM. Although the anti-Bcl-X antibodies used in our study detect both Bcl-XL and Bcl-XS, our data support the conclusion that Bcl-XL is the predominant isoform overexpressed in melanoma: (a) Bcl-XL was much more abundant than Bcl-XS based on Western blot studies; (b) very little if any Bcl-XS is typically found in normal tissues and tumors in vivo (37, 47); and (c) we have immunostained our tumors with a relatively Bcl-XS-specific antibody but observed no increased expression in melanoma.

Bcl-XL is a potentially important inhibitor of cell death in metastatic MM. Bcl-XL may be a stronger protector against apoptosis than Bcl-2 (55, 56), and interaction with Bax, a protein highly expressed in our cases of MM, is not required for Bcl-XL to exert its antiapoptotic effects (57). Prevention of hypoxia-induced cell death by Bcl-XL is particularly intriguing in the context of large solid tumors, where regions of profound hypoxia are not uncommon (58) and confer resistance to ionizing radiation (59) and some forms of chemotherapy (60). Exposure to low oxygen levels is associated with tumor progression in a mouse melanoma model (61), and hypoxia has been shown to select for tumors cells with diminished apoptotic potential (62).

![Western blot analysis of Bax, Bcl-2, Bcl-X, and Mcl-1 in benign melanocytic nevi and metastatic melanomas. Lanes 1 and 2, benign nevi; Lanes 3–12, metastatic MM samples.](image)

**Fig. 3** Western blot analysis of Bax, Bcl-2, Bcl-X, and Mcl-1 in benign melanocytic nevi and metastatic melanomas. Lanes 1 and 2, benign nevi; Lanes 3–12, metastatic MM samples. Left, protein molecular weights (in thousands).
In summary, this study demonstrates overexpression of Bcl-XL and Mcl-1 in human malignant melanoma. High levels of these apoptosis inhibitors may contribute to melanoma progression and to chemotherapy resistance. If future research confirms a role for Bcl-XL and Mcl-1 in melanoma drug resistance, then therapeutic strategies to selectively target and inactivate these proteins prior to chemotherapy may greatly improve treatment response and patient prognosis.

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