

# Bcl-xL and bcl-2 proteins in melanoma progression and UVB-induced apoptosis

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**Abstract.** Whether bcl-xL and bcl-2 play an essential role in melanoma progression and UVB-induced apoptosis is not completely understood. We investigated the expression of bcl-xL and bcl-2 in matched primary and metastatic melanoma tumors and melanoma cell lines from the same melanoma patients to clarify the importance of bcl-xL and bcl-2 in melanoma progression and in UVB-induced apoptosis. The expression of bcl-xL and bcl-2 proteins was examined by immunohisto(cyto)chemistry and Western blot in melanoma tumors and melanoma cells. Cellular viability and apoptosis were estimated after the melanoma cells were exposed to 30, 60 and 180 mJ/cm<sup>2</sup> UVB. Both primary melanoma tumors and melanoma cells showed lower expression of bcl-xL and bcl-2 proteins estimated as frequency of positive cells than their matched metastatic tumors and cells *in vitro*. After exposure to UVB, the cell viability decreased and the number of apoptotic cells increased in both primary and metastatic melanoma cell lines. These changes were more pronounced in the primary melanoma cells than in the matched metastatic cells. After UVB exposure, the expression of bcl-xL protein decreased in primary melanoma cells in a dose- and time-dependent manner, but the expression of bcl-2 was not influenced. The expression of bcl-xL and bcl-2 proteins was increased during melanoma progression from primary to metastatic melanoma. Reduction of bcl-xL, but not bcl-2 expression was involved in UVB-induced apoptosis in primary melanoma cells.

## Introduction

Cutaneous melanoma is the most aggressive form of skin malignancy. Experimental and epidemiological studies have revealed that genetic predisposition and UVB exposure play essential roles in melanoma carcinogenesis (1).

The bcl-2 family consists of a homologous network of genes regulating cell survival and apoptosis. Increased expression in

bcl-xL and bcl-2 has been shown to inhibit apoptosis, and formation of Bax heterodimers can counteract or modify the function of bcl-xL and bcl-2 to induce apoptosis (2). In addition, alterations of apoptosis related genes and their protein products have been considered to be involved in the development and progression of the majority of cancers. Generally, increased expression of the anti-apoptotic proteins, bcl-2 and bcl-xL, leads to aberrant cell proliferation and malignant growth (3). In head and neck squamous cell carcinoma, up-regulation of bcl-xL has been found to result in advanced tumors and poor prognosis (4) and in lung cancer, overexpression of bcl-2 correlates with a short survival (5). In addition, altered expression of bcl-xL and bcl-2 proteins may be involved in the development as well as progression of melanoma, as increased expression of bcl-xL and bcl-2 genes leads to an increased malignant potential caused by an inhibition of apoptosis and growth advantage for metastatic melanoma cells (6,7).

In normal skin melanocytes however, a redistribution of Bax protein from different compartments within the cells seem to be more important for the acceleration of apoptosis than a direct up-regulation (8). Bcl-xL has been shown to be critical in the protection of skin and epithelial cells from apoptosis (9), and down-regulation of bcl-xL protein increases the sensitivity of skin fibroblasts to UV-induced apoptosis (10). The application of bcl-xL and bcl-2 anti-sense oligonucleotides induces apoptosis in melanoma cells at different clinical stages (11). However, breast cancer cells with a high expression of bcl-xL are still susceptible to UV-induced apoptosis (12).

In the present study, we investigated the expression of bcl-xL and bcl-2 proteins in tumor tissue from both primary and metastatic melanomas from the same patients. The expression of these proteins in matched primary and metastatic melanoma cells was examined *in vitro* after UVB exposure.

## Materials and methods

**Antibodies, cell culture medium and chemicals.** Mouse monoclonal primary antibodies against bcl-xL, bcl-2 and  $\alpha$ -tubulin, and the secondary antibody conjugated HRP against mouse were from Santa Cruz (CA, USA). Protein blocker, mouse immunoglobulin, PAP and AEC ready-use agent were from DakoCytomation (Glostrup, Denmark). RPMI-1640 cell culture medium, fetal calf serum (FCS), L-glutamine (200 mM), penicillin (5000 IU/ml), streptomycin (5000  $\mu$ g/ml) and trypsin-EDTA were purchased from Life Technologies (Paisley,

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UK). 3,3-Diaminobenzidine tetrahydrochloride (DAB) was from Sigma Chemical Co. (MO, USA). Phosphate-buffered saline (PBS) and PBS-Tween (PBST) tablets were from EC Diagnostics AB (Uppsala, Sweden). Methanol and H<sub>2</sub>O<sub>2</sub> were from Merck (Darmstadt, Germany). Annexin V, propidium iodine (PI) and cell proliferation reagent WST-1 were from Roche Diagnostics GmbH (Mannheim, Germany).

**Melanoma tumors and cell lines.** This study was assessed by the Ethics Committee of Linköping University, Sweden. Seven matched primary and metastatic tissue samples from melanoma tumors in patients were collected from archived paraffin-embedded blocks at the Department of Clinical Pathology, Linköping University, Sweden. Sections of 5  $\mu$ m-thick were cut from the paraffin-embedded blocks. Matched primary (WM55P) and metastatic (WM55M1) melanoma cells were cultivated in RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin in an incubator with 5% CO<sub>2</sub> at 37°C.

**UV irradiation of melanoma cell lines.** UV irradiation has been proven to induce apoptosis in various types of cell. In this study, matched primary and metastatic melanoma cells were harvested from the original cell cultures by trypsinization and seeded in 35-mm cell culture dishes under the standard cell culture condition mentioned above for 24 h. The melanoma cells were then exposed to UVB in PBS (30, 60 and 180 mJ/cm<sup>2</sup>) as described previously (13). After UV exposure, the cultures were post-cultivated under standard cell culture conditions for up to 48 h.

**Expression analysis by immunohisto(cyto)chemistry.** Melanoma tissue sections were deparaffinized in xylene and rehydrated through series ethanol to Milli-Q water, and melanoma cells were fixed in 4% buffered formaldehyde after UVB exposure. The sections and melanoma cells were carefully rinsed in PBS and then pre-treated in methanol with 0.5% H<sub>2</sub>O<sub>2</sub> for 10 min and blocked with protein blocker for 60 min. The specimens were incubated with corresponding primary antibody at 4°C in a humid chamber overnight and, subsequently, with immunoglobulin (1:100 in PBS) for 60 min, and PAP (1:150 in PBS) for 60 min. The tumor sections were rinsed in PBS and positive cells were visualized by AEC ready-use agent, which gives a red colour for the positive staining distinguished from the pigment in the tissue. Since the melanoma cells did not contain any pigment, we were able to use DAB to show the positive cells. The cells were exposed to 0.05% DAB (brown colour) with 0.02% H<sub>2</sub>O<sub>2</sub> in PBS for 10 min. The specimens were slightly counterstained with haematoxylin for 1 min. Followed by a rinse in PBS, the samples were dehydrated through a serial ethanol to xylene and finally mounted. All procedures were performed at room temperature unless otherwise indicated. Samples known to give positive and negative immunostaining were included in each run. An additional negative control was performed in which the primary antibody was replaced by PBS and then taken through the same procedure. The positive samples were registered as - if <1% of the tumor cells were positive, + if 1-5%; ++ if 6-10% and +++ if >10% of the cells were positive.

Table I. Expression of bcl-xL and bcl-2 proteins in matched primary and metastatic melanomas.

Patients	bcl-xL		bcl-2	
	P	M	P	M
1	++	+++	+	++
2	-	++	-	+
3	+	+++	+	++
4	-	++	-	+
5	-	++	+	++
6	+	++	-	+
7	+	+++	+	++

**Expression analysis by Western blot.** After UVB irradiation and post-cultivation, melanoma cells were lysed in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% NaDOD, 0.1% SDS and 50 mM Tris pH 8.0). Total protein (10  $\mu$ g) was denatured at 95°C for 5 min and loaded in 12% ready gels (Bio-Rad Laboratories). The gels were run at 150 volt for 10 min and then 100 volt for 40 min. The separated proteins were transferred to PVDF membranes (Bio-Rad Laboratories) at 100 volt for 1 h. The membranes were blocked with 5% fat-free milk (Bio-Rad Laboratories) at 4°C overnight, and then were cut into two parts between the control protein ( $\alpha$ -tubulin) and the tested proteins. They were incubated with corresponding primary antibody (1:2000) in PBST at 20°C for 2 h and the corresponding secondary antibody (1:5000) conjugated HRP in 3% fat-free milk at 20°C for 1 h. The membranes were exposed to ECLplus at 20°C for 5 min and Hyper ECL film (Amersham Pharmacia Biotech). Expression levels of the proteins were represented as intensity of the bands as compared to the controls.

**Estimation of cellular viability and apoptosis.** Melanoma cells were subcultivated in 96-well cell culture plates at 37°C for 24 h. The medium was replaced by a thin layer of buffered PBS and exposed to UVB. The melanoma cells were then post-cultivated for up to 48 h. The absorbance of WST-1 was measured using an ELISA reader at 450 nm with a reference wavelength of 750 nm. Cell viability was represented as percentage of absorbance of the controls.

Apoptotic cells were detected by Annexin V. After UVB exposure and post-incubation, the melanoma cells were collected by trypsinization. In brief, the cell pellet was re-suspended in 250  $\mu$ l PBS and 100  $\mu$ l Annexin V/PI solution (20  $\mu$ l Annexin V in 1000  $\mu$ l of 10 mM Hepes buffer, 140 mM NaCl 5 mM CaCl<sub>2</sub> at pH 7.4 with 20  $\mu$ l of 50  $\mu$ g/ml PI) and incubated in the dark for 15 min. Subsequently, the signals from Annexin V and PI were simultaneously recorded from 10<sup>4</sup> cells in each sample by a flow cytometer (FACScalibur) from Becton-Dickinson (NJ, USA). The data was analyzed by using Becton-Dickinson CellQuest software.

## Results

**Expression patterns of bcl-xL and bcl-2 proteins.** Expression of bcl-xL and bcl-2 proteins was examined by immunohisto-

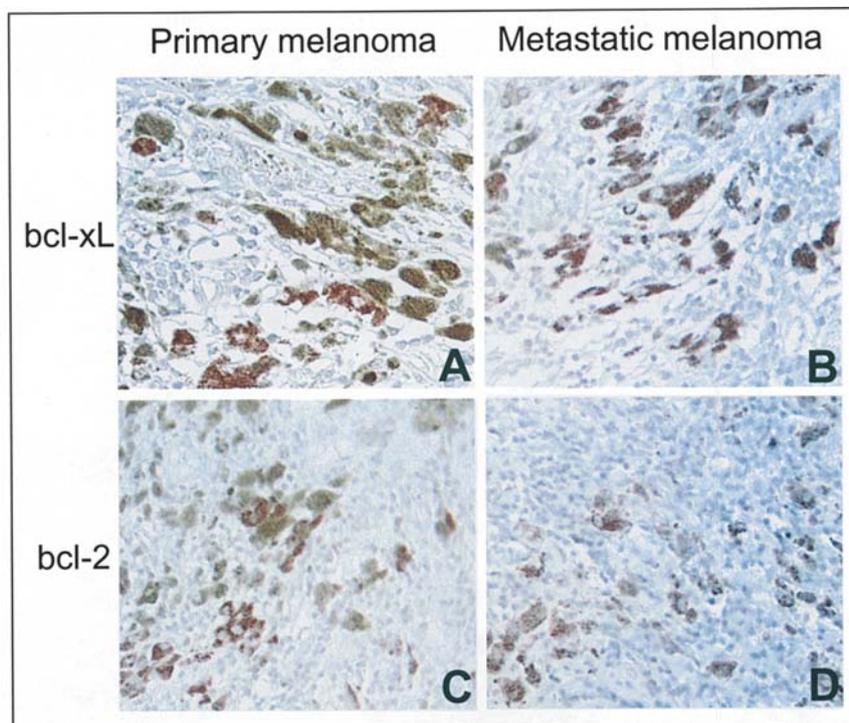


Figure 1. Expression of bcl-xL and bcl-2 proteins in matched primary and metastatic melanoma tumors. Expression of bcl-xL and bcl-2 proteins was examined by immunohistochemistry (AEC red color and melanin brown color) in patient 3 (Table I). The expression of both bcl-xL and bcl-2 proteins was found to be increased from the primary melanomas (A and C) to the matched metastatic melanoma tumors (B and D).

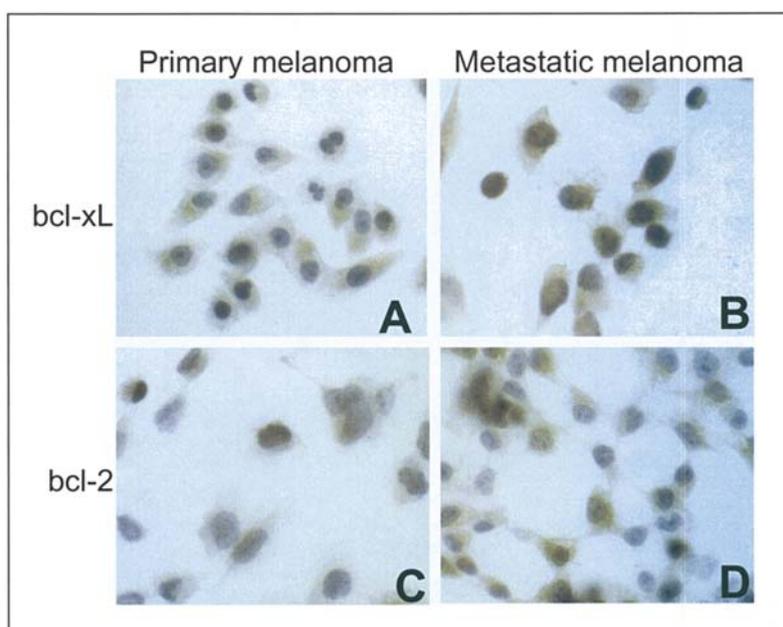


Figure 2. Expression of bcl-xL and bcl-2 proteins in matched primary and metastatic melanoma cell lines. Expression of bcl-xL and bcl-2 proteins was examined by immunocytochemistry (DAB brown color). The expression of both bcl-xL and bcl-2 proteins was found to be increased from the primary melanoma cell line (A and C) to the matched metastatic cells (B and D) established from the same patient.

chemistry in tumor tissue from matched primary and metastatic melanomas and in matched primary and metastatic melanoma cell lines. Expression of bcl-xL and bcl-2 proteins was found to be more pronounced, both in sections from metastatic melanomas (Table I and Fig. 1) and in cells from melanoma metastasis (Fig. 2), than in their matched primary melanomas.

*Cell viability and apoptosis after exposure to UVB.* Cell viability and apoptosis were examined in matched primary and metastatic melanoma cells after exposure to UVB in various doses and post incubation for different periods of time. After exposure to 30 or 60 mJ/cm<sup>2</sup> UVB and 24 h post-incubation the cell viability was 76 and 50% in the primary

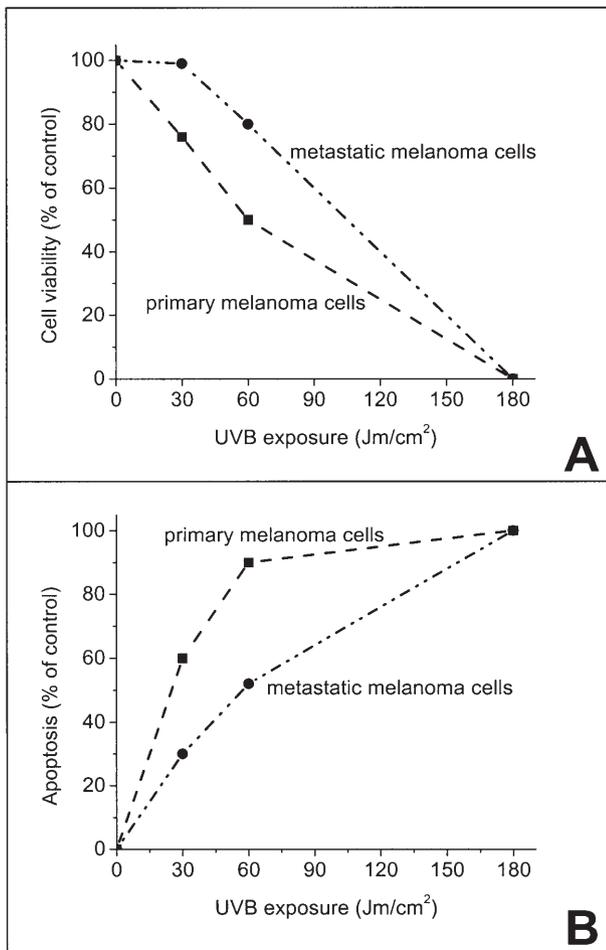


Figure 3. Cell viability and apoptosis in melanoma cells after UVB exposure. The viability decreased in both primary and metastatic melanoma cells after UVB exposure at various doses and 24 h post-incubation (A). The number of apoptotic cells increased after UVB exposure (B). The decrease in cell viability and increase in apoptosis were UVB dose-dependent. The primary melanoma cells were more vulnerable to UVB exposure than the matched metastatic cells.

melanoma cells, and 99 and 80% in the matched metastatic melanoma cells. The reduction in cell viability was UVB dose-dependent and all cells from both primary and metastatic melanomas were dead when they were exposed to 180 mJ/cm<sup>2</sup> UVB. Primary melanoma cells were more vulnerable to UVB exposure than the matched metastatic melanoma cells (Fig. 3A).

Following UVB exposure, the number of apoptotic cells in both primary and metastatic melanoma cell lines was markedly increased in a dose-dependent manner. After exposure to 30 and 60 mJ/cm<sup>2</sup> UVB and incubation under standard cell culture conditions for 24 h, the number of apoptotic cells in the primary melanoma was 60 and 90%. Corresponding figures in its matched metastatic melanoma cells were 30 and 52%. The increase in apoptotic cells was UVB dose-dependent. The primary melanoma cells were more sensitive to UVB-induced apoptosis than the matched metastatic melanoma cells (Fig. 3B).

*Expression of bcl-xL and bcl-2 after UVB exposure.* After exposure to 30 and 60 mJ/cm<sup>2</sup> UVB (Fig. 4) and post-

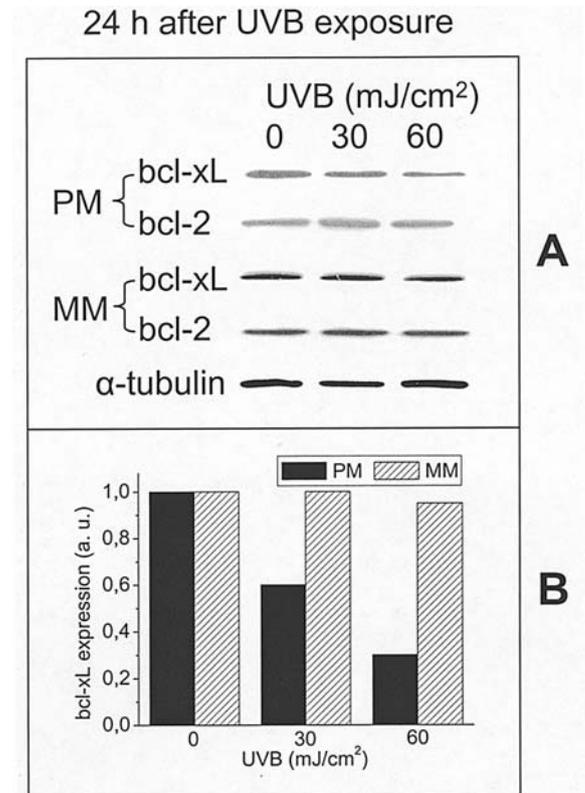


Figure 4. Expression of bcl-xL and bcl-2 proteins 24 h after UVB exposure at various doses. Expression of bcl-xL and bcl-2 proteins was examined by Western blot in matched primary melanoma (PM) and metastatic melanoma cells (MM). The expression of bcl-xL gradually decreased after UVB exposure. This decrease was not observed in the matched metastatic melanoma cells. The expression of bcl-2 protein was not notably influenced by UVB exposure in primary or metastatic melanoma cell lines.  $\alpha$ -tubulin was used as an internal control for the protein loading (A). The expression levels of bcl-xL were presented as arbitrary units (a.u.) (B).

incubation for different periods of time (Fig. 5), the expression of bcl-xL and bcl-2 proteins was examined by Western blotting in matched primary and metastatic melanoma cell lines. In the primary melanoma cells, the expression of bcl-xL protein gradually decreased in a UVB dose- and post-incubation time-dependent manner. However, the expression levels of bcl-xL in the matched metastatic melanoma cells and bcl-2 proteins in both primary and metastatic melanoma cells were not markedly changed.  $\alpha$ -tubulin was used as an internal control. The response of bcl-xL protein in the primary melanoma cells was confirmed by immunocytochemistry after UVB exposure (Fig. 6).

## Discussion

In the present study, the expression of bcl-xL and bcl-2 proteins was examined in matched primary and metastatic melanoma tumors and in melanoma cell lines. We showed by immunostaining and Western blotting that the expression of both bcl-xL and bcl-2 proteins was higher in metastatic melanomas than in their matched primary melanomas. Many molecules have been considered to be involved in melanoma progression in different stages. p16INK4A is a candidate tumor suppressor, frequently deleted in melanoma (14). MAP kinase proteins ras,

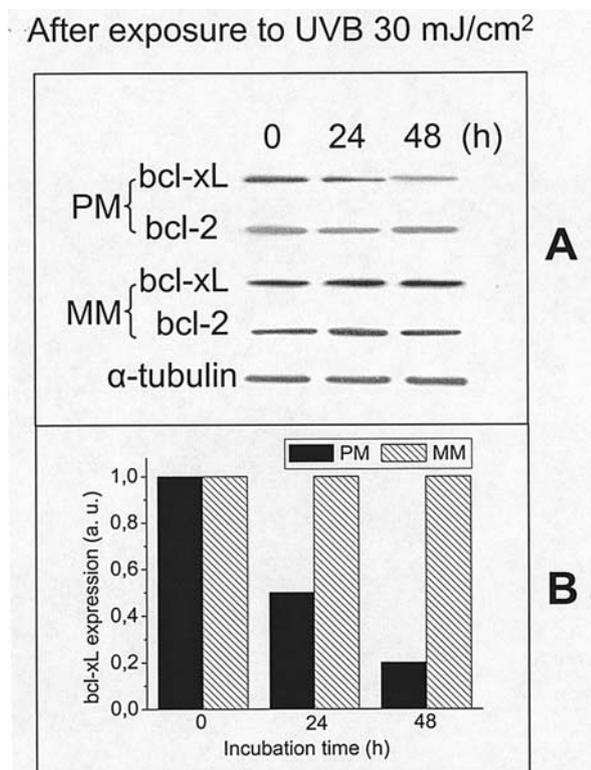


Figure 5. Expression of bcl-xL and bcl-2 proteins after UVB exposure and post-incubation for various periods of time. The expression of bcl-xL and bcl-2 proteins was examined by Western blotting in matched primary melanoma (PM) and metastatic melanoma cells (MM). The expression of bcl-xL gradually decreased by prolonging the post-incubation time after UVB exposure (30 mJ/cm<sup>2</sup>). This decrease was not observed in the matched metastatic melanoma cells. The expression of bcl-2 protein was not notably influenced.  $\alpha$ -tubulin was used as an internal control for the protein loading (A). The expression levels of bcl-xL were presented as arbitrary units (a.u.) (B). The expression of bcl-xL and bcl-2 proteins in the primary melanoma cells was examined by immunocytochemistry. The expression of bcl-xL was decreased after exposure to UVB 30 Jm/cm<sup>2</sup> and post-cultivation for 24 h (C). The expression of bcl-xL decreased after UVB irradiation (right panel) as compared to the control (left panel).

raf and erk are also involved in melanoma development and progression (15). Much remains to be learned about the pathogenesis of melanoma. We provide evidence that bcl-xL and bcl-2 proteins in the bcl-2 pathway play an important role in progression of melanoma from primary to metastasis.

When the expression of bcl-xL and bcl-2 proteins in matched primary and metastatic melanoma cells was examined, we found the level of bcl-xL protein to decrease in a UVB dose- and post-incubation time-dependent manner. The expression of bcl-xL protein in the matched metastatic melanoma cells was more resistant and was not changed by the same UVB dose. Moreover, the expression of bcl-2 protein in either primary or metastatic melanoma cells was not influenced by UVB exposure. These data indicate that bcl-xL played an important role in UVB-induced apoptosis in primary melanoma cells but not in matched metastatic cells. Although increased expression in bcl-xL and bcl-2 played a role in melanoma progression, bcl-2 was not considered as a key protein in the UVB-induced apoptosis in these melanoma cell lines. The induction of apoptosis might be due to the function of other apoptosis-related factors and pathways.

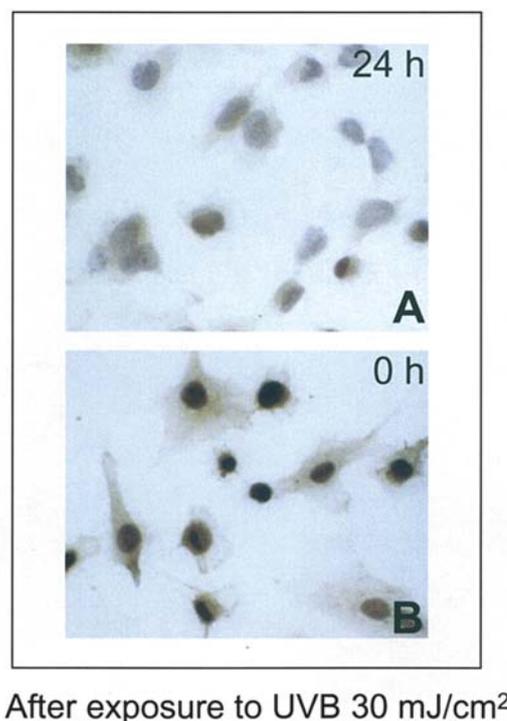


Figure 6. The expression of bcl-xL protein after UVB exposure and post-incubation. The decreased expression of bcl-xL in the primary melanoma cells was examined by immunocytochemistry after 30 mJ/cm<sup>2</sup> UVB and 24 h post-incubation (A) as compared to the control, which was examined directly after UVB exposure (B).

Melanoma cells have been demonstrated to resist apoptosis mediated through the mitochondrial pathway (16), especially in the advanced stages of melanoma. Because systemic therapies for advanced melanoma have limited effectiveness, the search for a more active therapy against advanced melanoma is important. In this study, we found that both bcl-2 and bcl-xL were increased during melanoma progression from primary to metastatic melanoma. This indicated that these proteins were indeed involved in melanoma progression to provide melanoma growth advances. We have previously shown that UVA and UVB induce apoptosis in human skin melanocytes via different pathways. UVA enhances protein expression related to cell growth, such as p73 and Nup88 proteins. UVB has been proven to up-regulate proteins related to cellular proliferation, such as Id1 and p27 (13). In a study of UVB on wild-type p53 and mutant p53 melanoma cells, we found that one mechanism of UVB-induced apoptosis is through the expression of survivin protein in a p53-dependent manner. The expression of survivin is markedly decreased in wild-type p53 melanoma cells but not in mutant p53 melanoma cells (17).

In this study, UVB was used as an apoptosis inducer to trigger apoptosis in melanoma cell lines. Primary melanoma cells were more vulnerable to UVB-induced apoptosis than their matched metastatic melanoma cells. The UVB-induced apoptosis depended on the expression of bcl-xL protein. This result indicated that a high level of bcl-xL in melanoma cells might cause a resistance to cancer therapy. By passing the bcl-xL pathway with antisense treatment against bcl-xL in combination with chemotherapy might be a new therapeutic strategy for advanced melanoma.

In conclusion, both bcl-xL and bcl-2 proteins were involved in melanoma progression from primary and metastatic tumors. Reduction of bcl-xL but not bcl-2 protein expression played an important role in UVB-induced apoptosis in primary melanoma cells.

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