Bcl-X$_L$ protein is markedly decreased in UVB-irradiated basal cell carcinoma cell lines through proteasome-mediated degradation

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Abstract. There is considerable evidence that the excessive ultraviolet radiation B (UVB) from sunlight is implicated in skin damage, ultimately inducing the death of keratinocytes. The UVB-induced apoptotic pathways are tightly regulated by the balance between pro-apoptotic and anti-apoptotic molecules. Among them, modulations of Bcl2 family proteins are important to decide the fate of UVB-irradiated cells. If the apoptotic pathway does not work properly, the damaged cells have a chance to transform into a carcinoma, such as basal cell carcinoma or squamous cell carcinoma of the skin. To develop a strategy of inducing apoptosis of skin cancer cells, the current study was performed to investigate the apoptotic pathway, especially focused on Bcl2 family proteins, in curcumin or UVB-treated basal cell carcinoma cell lines. Our data showed that the decreased proliferation rates and apoptotic DNA laddering were clearly observed in UVB irradiation, but not markedly observed in curcumin treatment. The decreased expression of Bcl-X$_L$, which is involved in protection of apoptosis, was also clearly observed in UVB-irradiated cells without markedly changing mRNA levels. However, the expression of Bax or Bcl2 were not markedly changed by UVB-irradiation. The decreased expression of Bcl-X$_L$ protein after UVB treatment was partially restored in the presence of MG132, which is an inhibitor of proteasome, implying that the down-regulation of Bcl-X$_L$ may be regulated by the proteasome-mediated degradation. Our data demonstrated that the expression of Bcl-X$_L$ protein was decreased by proteasome-mediated degradation prior to change of mRNA level in UVB-induced apoptotic basal cell carcinoma cell lines, thereby these results will offer fundamental information to develop a strategy of inducing apoptosis of skin cancer cells.

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Introduction

Basal cell carcinoma (BCC) is one of the most commonly encountered neoplasms in the world and is characterized as locally aggressive with little metastatic potential (1). Due to poor ability of metastasis, it is a good candidate to develop a strategy of inducing apoptosis for treatment.

Ultraviolet B (UVB) is a well-known major risk factor for the development of skin cancer in epidermis (2). The UVB-damaged cells should permit the apoptotic pathways for preventing cancer development. If some damaged cells overcome the apoptotic pathway, the cells have a good chance to transform into a cancer. The BCC showed up-regulation of Bcl2, which plays a role as an anti-apoptotic protein in cells, and the increased Bcl2 expression has strengthened the resistance against apoptosis (3,4). Owing to this molecular change, analysis of apoptotic pathway, especially focused on Bcl2 family proteins, may be a key step to develop a strategy for inducing apoptosis in BCC.

Thus to develop the strategy of inducing apoptosis in localized BCC, we investigated the expression of Bcl2 family proteins in BCC cell lines after chemopreventive agent (curcumin) treatment or UVB irradiation.

Materials and methods

Materials. Antibodies against Bcl2, Bcl-X$_L$, Bax, PARP and CPP32 were obtained form Santa Cruz Biotechnology (Santa Cruz). Curcumin and MG132 were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture. Human basal cell carcinoma cell lines were established as described previously (5). The cell lines were maintained at 37˚C in a humidified atmosphere of 95% air and 5% CO$_2$ in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin. For experiments, cells (5x10^4 cells/ml) were seeded in culture dish, and maintained in the tissue culture incubator.

UVB irradiation. UVB was supplied by a closely spaced array of seven Westinghouse FS-40 sunlamps, which delivered uniform irradiation at a distance of 38 cm. The
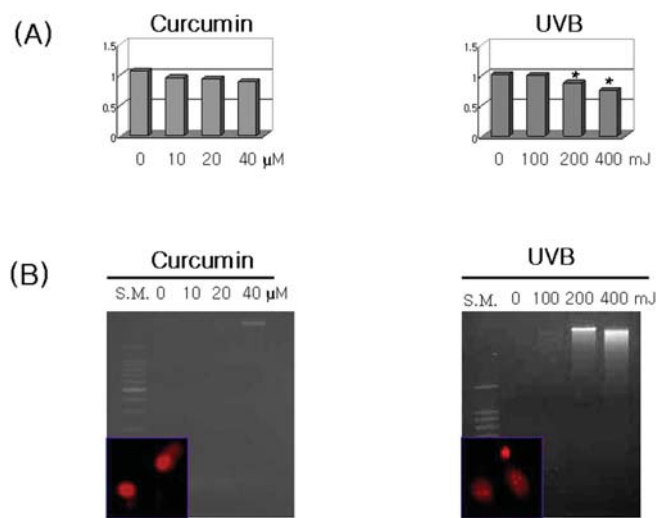
Figure 1. Decreased cell proliferation rates concomitant with distinct DNA laddering in UVB-irradiated BCC cell lines, compared to curcumin-treated BCC cell clines. Cells were treated by various doses of curcumin or UVB for 3 days. The viable cells were counted daily using trypan blue assay (A). The cytosolic DNA were extracted 3 days after curcumin or UVB treatment, and analyzed on a 2% agarose gel (B). Experiments were repeated 2 times and similar results were obtained. Distinct nuclear fragment were observed in UVB-irradiated BCC cell lines by fluorescence microscope (x400, Box). (S.M., size marker).
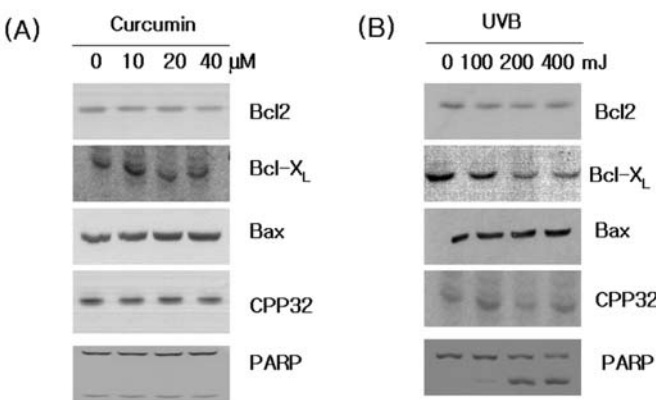

Figure 2. Distinct expression of apoptosis-related protein profiles between curcumin (A) or UVB-irradiated BCC cell lines (B). Cells were treated by various doses of curcumin or UVB, and then the expression of apoptosis-related proteins were analyzed by Western blotting 24 h after treatment. The expression of Bcl-X_L and PARP were markedly distinguished between two conditions. Experiments were repeated 2 times and similar results were obtained.

Figure 3. Comparison of Bcl2 and Bcl-X_L mRNA between curcumin or UVB-treated BCC cell lines. Total RNA were extracted 24 h after curcumin (A) or UVB treatments (B). The Bcl-2 and Bcl-X_L mRNA were specifically amplified by RT-PCR using specific primers and then amplified products were analyzed by 1.2% agarose gel electrophoresis. Experiments were repeated 2 times and similar results were obtained.
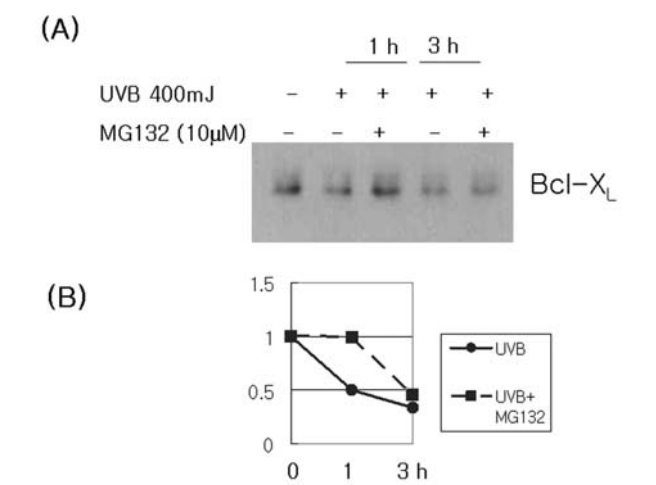
Figure 4. Decreased expression of Bcl-X_L was partially attenuated under MG132 in UVB-irradiated BCC cell lines. Cells were co-treated with MG132 (10 μM) and UVB (400 mJ), and then analyzed by Western blot analysis using anti-Bcl-X_L antibody (A). The expression patterns of Bcl-X_L are presented as a schematic graph (B). Experiments were repeated 2 times and similar results were obtained.

energy output of UVB (290-320 nm) at 38 cm was measured with a UVB photometer (IL1350 photometer, International Light, Newburyport, MA). Cells were exposed to doses of 0 and 400 mJ/cm². To prevent light absorption by tissue-culture medium, the culture medium was removed just prior to irradiation and replaced with a thin layer of phosphate-buffered saline (PBS) to cover the cells. After UVB irradiation, cells were fed with fresh growth medium.

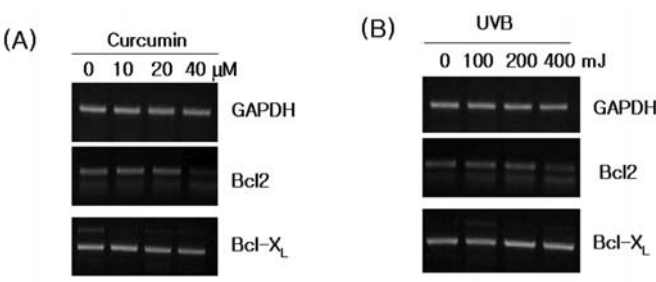*DNA fragmentation assay.* For DNA fragmentation assay, cells were harvested, washed in PBS and then lysed in a digestion buffer containing 50 mM Tris (pH 8.0), 0.5% sarkosyl, 0.5 mg/ml proteinase K, and 1 mM EDTA at 55˚C for 3 h. RNase A (0.5 μg/ml) was added and further incubated at 55˚C for 18 h. The genomic DNA was extracted by phenol-chloroform-isoamylalcohol and analyzed by 1.7% agarose gel electrophoresis. The DNA was visualized and photographed under ultra-violet illumination after staining with ethidium bromide.

Assessment of apoptotic nuclei. Cells were fixed with 95% cold-ethanol for 4 min and washed twice with PBS. After fixation, cells were stained with staining solution [propidium iodide (PI) (1 μg/ml), RNase A (0.1 mg/ml)] for 20 min at room temperature. Nucleor morphology of PI-stained cells was observed under fluorescence microscope (Olympus Optical, Tokyo, Japan).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from cells using the RNAzol™ B
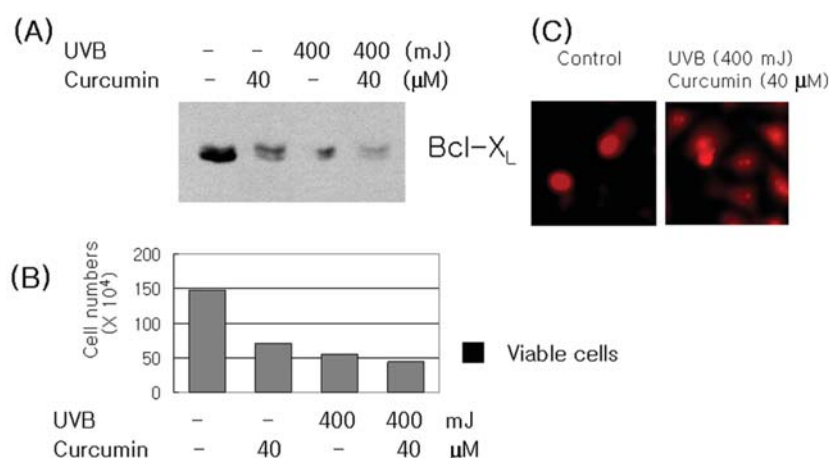
Figure 5. Co-treatment with curcumin and UVB radiation induced markedly decreased expression of Bcl-X$_L$. Cells were co-treated with curcumin (40 μM) and UVB (400 mJ), and then the expression Bcl-X$_L$ analyzed by Western blot analysis using anti-Bcl-X$_L$ antibody (A). The expression patterns of Bcl-X$_L$ are presented as a schematic bar graph (B). Experiments were repeated two times and similar results were obtained.

(Biotecx laboratories, Houston, TX) according to the manufacturer's instructions and quantitated by spectrophotometer. One microgram of total RNA was reverse transcribed using M-MLV reverse transcriptase (Promega Co., Madison, WI). The PCR reaction was carried out under the conditions recommended by the manufacturer's instructions (Takara Co., Otsu, Japan). The primer sequences and product sizes were as follows: i) GAPDH (forward; 5'-CGTCTTCACCAC CATGGAGA-3', reverse; 5'-CGGCCATCACGCCACAG TTT-3'), ii) Bcl-X$_L$ (forward; 5'-CCCAGAAAGGATACA GCTGG, reverse; 5'-CTCCTGGATCCAAGGTCTA), iii) Bcl2 (forward; 5'-TTTGAGTTCGGTGGGGGTCAT-3', reverse; 5'-TGACTTCACTTGTGGCCCAG-3').

Western blot analysis. Cells were lysed in lysis buffer [10 mM Tris (pH 7.4), 5 mM EDTA, 130 mM NaCl, 1% Triton X-100, PMSF (10 μg/ml), aprotinin (10 μg/ml), leupeptin (10 μg/ml), 5 mM phenanthroline, and 28 mM benzamidine-HCl] for 30 min on ice. Lysates were clarified by centrifugation. Lysates were quantitated using the Bradford assay (Life Science Co., CA, USA) with bovine serum albumin as a reference standard. Proteins (35 μg) were resolved by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gels) and transferred to immobilon-P transfer membrane (Millipore Co., MA, USA). After incubation with primary antibodies, proteins were visualized by incubation with horseradish peroxidase-conjugated secondary antibodies, followed by ECL according to the manufacturer's instructions (Amersham Life Science Co., UK).

Results

UVB induces decreased cell proliferation rates in BCC cell lines. To study the effect of UVB and curcumin on cell proliferation rates, BCC cell lines were exposed to UVB and curcumin at doses ranging from 100 to 400 mJ/cm^2 and from 10 to 40 μM concentrations, respectively. The viable cells were counted using trypan blue exclusion method. As shown in Fig. 1A, the number of viable cells was decreased in a dose-dependent manner of UVB irradiation. The proliferation

rates of UVB-irradiated BCC cell lines on 400 mJ/cm^2 dose were decreased ~30% compared to non-irradiated cells. However, the proliferation rates of curcumin-treated BCC cell lines were not markedly decreased compared to those of UVB-irradiated BCC cell lines. Consistently, the distinct cytosolic DNA fragments from UVB-irradiated BCC cell lines were observed by agarose gel electrophoresis, but not in curcumin-treated BCC cell lines (Fig. 1B). We confirmed the fragmented nucleus in UVB-irradiated BCC cell lines by fluorescence microscope (Fig. 1B; box).

Alteration of Bcl2 family proteins in UVB-irradiated BCC cell lines. To study the effect of UVB and curcumin on the expression of Bcl2 family proteins, BCC cell lines were exposed to UVB and curcumin at doses ranging from 100 to 400 mJ/cm^2 and from 10 to 40 μM concentrations, respectively. The expression of Bcl2, Bcl-X$_L$, Bax, CPP32 and PARP were analyzed 24 h after treatment by Western blotting (Fig. 2). When BCC cell lines were treated by curcumin, the expression of Bcl2 and Bcl-X$_L$ were not markedly decreased (Fig. 2A). However, in case of UVB-irradiation, the expression of Bcl-X$_L$, but not Bcl2, were clearly decreased in a dose-dependent manner (Fig. 2B). However, the expression of Bax and CPP32 showed similar patterns between curcumin and UVB treatment. PARP cleavages were only detected in UVB-irradiated BCC cell lines (Fig. 2). Interestingly the levels of Bcl2 and Bcl-X$_L$ mRNA had distinct patterns in the two conditions (Fig. 3). The expression of Bcl2 mRNA was decreased in a dose-dependent manner in curcumin and UVB treatment. However, the expression of Bcl-X$_L$ was not changed by curcumin or UVB treatment, implying the modulation of Bcl-X$_L$ may be regulated at post-translational level.

To confirm whether the Bcl-X$_L$ protein is degraded by proteasome mediated pathway in UVB (400 mJ/cm^2)-irradiated BCC cell lines, we analyzed the degradation degrees of Bcl-X$_L$ in the presence or absence of MG132 (10 μM, proteasome inhibitor) after UVB irradiation (Fig. 4). The down-regulation of Bcl-X$_L$ in UVB-irradiated BCC cell

lines were partially attenuated by the treatment of MG132, implying decreased expression of Bcl-X_L by UVB treatment may be initially mediated by proteasome degradation pathway.

Synergistic down-regulatory effect of curcumin and UVB on Bcl-X_L expression. Next, we studied the synergistic effect of curcumin and UVB on down-regulation of Bcl-X_L in UVB-irradiated BCC cell lines. The expression of Bcl-X_L was more decreased by co-treatment of UVB (400 mJ/cm^2) and curcumin (40 μM) than each treatment (Fig. 5). Consistent with this result, the rates of cell death were increased by co-treatment of UVB and curcumin (data not shown).

Discussion

Apoptosis is a critical mechanism for preventing the accumulation of cells with malignant potential. In several cell types, apoptosis relies on the mitochondrial contribution to amplify the death signal (6). The mitochondrial network triggers the release of cytochrome c from mitochondria, ultimately resulting in stimulation of caspase 3 (7,8). Recently, it has been recognized that the Bcl2 family of proteins is a critical mitochondrial intracellular checkpoint. Actually, the ratio of anti-apoptotic (Bcl2, Bcl-X_L, MCL1) to pro-apoptotic (Bim, Bad, Bax, Bak) molecules contributes to set the threshold of susceptibility to apoptosis. Biochemical and physiological regulations of these two groups of proteins determine whether a cell survives or undergoes apoptosis (9). Furthermore, some Bcl2 family members have dual roles, serving as molecular mediators of both apoptosis and cell cycle progression. The functional significance of Bcl2 family members in linking apoptosis and the cell cycle continues to empower the pharmacological quest for therapeutics in cancer (10-14).

As our understanding of cancer molecular biology increases, success has been achieved in the development of therapies that target malignancies associated with the overexpression of anti-apoptotic proteins. Johnson *et al* reported that melanoma cells were radiosensitized through combined inhibition of protein regulators of cell survival, namely suppression of cFLIP and Bcl-xL expression (15).

In this study, the Bcl-X_L protein was markedly decreased by UVB irradiation in BCC cell lines. Our data also indicate that the proteasome-mediated degradation may be partially involved in the initial down-regulation of Bcl-X_L without regulation of transcriptional level. If the initial down-regulation of Bcl-X_L is impaired by a molecular event, this is a chance for the cell to escape apoptosis and eventually develop cancer. In contrast, modulation of Bcl-X_L by inhibitor or anti-sense oligonucleotides of Bcl-X_L engenders apoptotic cell death in tumor cells. When the Bcl-X_L-overexpressing mesothelioma was treated by small molecule Bcl-X_L inhibitor such as 2-methoxy antimycin A3 alone, or in the combination with other chemotherapeutics, the cancer cells showed increased chemosensitivity and apoptotic cell death *in vitro* and *in vivo* (16). In addition Yamanaka *et al* reported that bispecific antisense oligonucleotide targeting Bcl2 and Bcl-X_L genes can induce apoptosis and enhance-ment of chemosensitivity in human prostate cancer LNCaP cells (17). We also need further study to evaluate the effect of Bcl-X_L antisense oligonucleotide or inhibitor on apoptosis of Bcl-X_L over-expressing skin cancer.

Thus, our data demonstrated that the expression of Bcl-X_L protein was decreased by proteasome-mediated degradation prior to change of mRNA level in UVB-induced apoptotic basal cell carcinoma cell lines, thereby these results will offer fundamental information to develop a strategy of inducing apoptosis of skin cancer cells through modulation of Bcl-X_L expression.

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