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# Anti-apoptotic effect by Bcl-2 in UVB-irradiated keratinocytes.

Hidetoshi Takahashi[1*], Masaru Honma[1], Akemi Ishida-Yamamoto[1], Kazuhiko Namikawa[2], Akiko Miwa [3,4], Haruo Okado [3,4], Hiroshi Kiyama[2,4], Hajime Iizuka[1]

[1]Departments of Dermatology, Asahikawa Medical College, Asahikawa, 078-8510 Japan

[2]Departments of anatomy, Asahikawa Medical College, Asahikawa, 078-8510 Japan

[3]Department of Neurobiology, Tokyo Metroporitan Institute for Neuroscience, Fuchu, Tokyo, 183-8526 Japan

[4]Core Research for Evolution Science and Technology (CREST), Japan Science and Technology, Kawaguchi, Saitama, 332-0012 Japan

Bcl-2 is a member of large bcl-2 family and protects cells from apoptosis. Using bcl-2-expressing adenovirus vector (Ad-bcl-2), we investigated the effect of bcl-2 on UVB-induced apoptosis. Adenovirus vector efficiently introduced bcl-2 gene in cultured normal mouse keratinocytes (NMK cells); almost all NMK cells ($1x10^6$) were transfected at $1x10^8$ PFU/ml. Bcl-2-transfected NMK cells were significantly resistant to UVB-induced apoptosis with the suppressive effect dependent on bcl-2-expression level. Following UVB irradiation caspase 8, 3, 9 activities were stimulated in NMK cells, while in bcl-2-transfected cells, only caspase 8, but not caspase 3 or 9 activities were stimulated. In order to investigate the effect of bcl-2 *in vivo*, topical application of Ad-bcl-2 on tape-stripped mouse skin was performed. Following the application, Bcl-2 was efficiently overexpressed in almost all viable keratinocytes. The expression was transient with the maximal expression of Bcl-2 at 1st day following the application of $1x10^9$ PFU in 200ml. The introduced Bcl-2 remained at least for 6 days. UVB irradiation (1250 J/$m^2$) induced apoptosis within 12 h and the maximal effect was observed at 24 h in control mouse skin. Bcl-2-transfected mice skin were resistant to UVB-induced apoptosis. Topical application of empty adenovirus vector alone had no effect on Bcl-2 expression or UVB-induced apoptosis. These results indicate that adenovirus vector is an efficient gene delivery system into keratinocytes and that Bcl-2 is a potent inhibitor of UVB-induced apoptosis both *in vitro* and *in vivo*.

**Key words**: adenovirus, apoptosis, bcl-2, keratinocytes, UVB

## INTRODUCTION

Ultraviolet B (UVB) irradiation produces "sunburn cells" in the epidermis (1). The sunburn cells are keratinocytes showing eosinophilic cytoplasm with or without remnants of shrunken and condensed nuclei in hematoxylin and eosin staining. Biochemical analyses revealed that UVB-irradiated keratinocytes show DNA fragmentation accompanied with sequential caspase activation (2). At present, sunburn cells are regarded as a specific type of apoptosis, that is triggered by DNA damage.

Bcl-2 was originally identified on the basis of gene activation by chromosomal t(14:18) translocation in non-Hodgkin B cell lymphoma (83. Molecular studies revealed that Bcl-2 is a member of large Bcl-2 family and protects many types of cell from apoptosis (4). In the epidermis, Bcl-2 protein is expressed in the basal cell layer (5), UV irradiation decreases Bcl-2 expression of rat skin *in vivo*, and Bcl-2 induced apoptosis (6). However, the precise function of Bcl-2 is still unknown. In the present study, using adenovirus vector carring bcl-2 (Ad-bcl-2), we investigated the effect of bcl-2 protein on UVB-induced apoptosis of keratinocytes both in vitro and in vivo.Corresponding author: Hidetoshi Takahashi, M.D.

* To whom correspondence should be addressed.
E-mail address: ht@asahikawa-med.ac.jp

## MATERIALS AND METHODS

***Cell culture*** Primary epidermal cells were prepared from newborn BALB/c mice according to the methods described by Yuspa (7) and were cultured in keratinocyte growth medium (KGM) containing epidermal growth factor (10 ng/ml), insulin (5 mg/ml), and bovine pituitary extract (50 mg/ml) at 37 °C in 5% $CO_2$ atmosphere.

***Recombinant Adenovirus vector carrying bcl-2 (Ad-bcl-2) and transfection into cultured normal mouse keratinocytes (NMK)*** All viruses described above were grown in 293 cells and purified by $CsCl_2$ gradient centrifugation. Infection was performed by adding recombinant adenovirus (titer ratio; AxCALNLBcl-

2:AxCANCre = 10 : 1) to KGM medium. The cells were incubated at 37°C for 60 min. The medium was changed to fresh KGM medium, and the cells were incubated at 37°C for 24 h before UVB irradiation.

***UVB irradiation and Cell Death Assay*** NMK cells were seeded at 1 X $10^4$ cells in a 96-well microtiter plate and were washed twice with phosphate-buffered saline (PBS, pH 7.5). UVB irradiation source was a Toshiba-Eizai Dermaray instrument (DMR-1, Tokyo, Japan) equipped with five fluorescent lamps (FL-20-SE-30, Toshiba, Japan). Cells were stained for 20 min at room temperature with 0.75% crystal violet in 50% ethanol, 0.25% NaCl, and 1.75% formaldehyde, and were washed three times with PBS. Dye uptake was quantified by measuring optical density (OD) at 540 nm using an automated Micro-ELISA autoreader.

***Caspase activity assay*** Caspase assays were carried out using fluorogenic substrates, according to the protocol provided by the manufacturer (Kamiya Biomedical Co., Seattle, WA). The reaction mixtures were incubated at 30°C for 1 h, and fluorescence was measured by a fluorometer with an excitation of 400 nm and emission of 505 nm.

***Western blot analysis*** The blots were blocked with 5% skim milk in Tris-buffered saline (TBS, pH 7.6) for 1 h at room temperature, and then were incubated at 4°C overnight with anti-Bcl-2, anti-Bax, anti-Bcl-xL, anti-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), that had been diluted 1,000-fold in TBS. After washing at room temperature with 0.1% Tween-20 in TBS, a blotting detection kit for rabbit antibody (Amersham International plc, UK) was used for immunodetection.

***Animal and topical gene transfer*** Female hairless mice (HOS:Hr-1) aged 6 weeks were purchased from Shizuoka Laboratory Center (Shizuoka, Japan). Mice were anaesthetised with sodium pentobarbital (350 mg/kg). Adenovirus virus transfer into mice skin was performed according to the method by Lu et al (8).

***Immunostaining of bcl-2*** The frozen sections of mice skin were pretreated with 0.3% hydrogen peroxide solution to block endogenous peroxidase activity. Following incubation with normal goat serum diluted in PBS for 10 min, the sections were incubated with anti-bcl-2 antibody diluted 1:200 in PBS for 2 h at room temperature. The sections were washed with PBS, followed by application of Histofine streptavidine-biotin (SAB)-PO(M) kit (Nichirei, Tokyo, Japan).

***Apoptotic cell counting*** Biopsy specimens were taken, processed routinely, and stained with hematoxylin and eosin. The number of apoptotic cells in the epidermis was counted and expressed as number of cells per cm (9).

## RESULTS

**Adenovirus vector efficiently introduces bcl-2 into cultured NMK cells.** The transfection efficacy increased in a concentration-dependent manner with the maximal transfection observed at $1x10^8$ PFU/ml. At that concentration, almost 100% of NMK cells were transfected by Ad-bcl-2 .

**Bcl-2-transfected NMK cells are resistant to UVB-induced apoptosis.** The maximal effect of UVB was observed at 600J/$m^2$, where less than 20% of cells survived. Bcl-2-transfected cells were resistant to UVB-induced apoptosis and more than 80% of bcl-2 transfected NMK cells survived. The anti-apoptotic effect was parallel to bcl-2 expression level and the maximal effect was observed at $1x10^8$ PFU/ml. Bax and Bcl-xL expression levels were not altered by the transfection of Ad-bcl-2 into NMK cells.

**Activities of caspase 3 and 9, but not of caspase 8, are suppressed in UVB-irradiated bcl-2-transfected NMK cells.** Following UVB irradiation, caspase 3, 9, and 8 activities were markedly stimulated. Transfection of bcl-2 significantly inhibited the UVB-induced caspase 3 and 9 activities. In contrast, caspase 8 activity was not affected by bcl-2-transfection and marked stimutation of caspase 8 was detected in bcl-2-transfected NMK cells following UVB irradiation .

**Ad-bcl-2 efficiently introduced bcl-2 into mouse skin** *in vivo.* After 24 h of virus exposure, we observed that $1x10^9$ PFU (200 ml) of Ad-bcl-2 induced maximal transfection of Bcl-2 into mouse skin. Bcl-2 was highly expressed in the epidermis at 24 h and remained at least for 6 days. At 9th day, the Bcl-2 expression returned to basal level. Histochemical study revealed that Bcl-2 was directly transfected in the suprabasal layers of the epidermis. In contrast, Bcl-2 expression was restricted to the basal cell layer of nontreated skin suggesting that the gene transfer using adenovirus vector primarily affects the suprabasal cell layers.

**Bcl-2-transfected mouse epidermis is resistant to UVB-induced apoptosis *in vivo*.** UVB (1250 J/$m^2$) was irradiated at 2nd day following adenovirus application of $1x10^9$ PFU in 200 ml on mouse skin. UVB irradiation induced apoptosis within 12 h and the maximal induction was observed at 24 h. The induction of apoptosis was significantly suppressed in the Bcl-2-transfected skin compared to nontreated skin.

## DISCUSSION

Our results clearly demonstrated that Bcl-2 protects keratinocytes from UVB-induced apoptosis both *in vitro* and *in vivo*. Previously Haake et al reported that stratified epidermis, which is constructed by Bcl-2-transfected HaCaT cells is resistant to UV-induced apoptosis (6). Recently, Bcl-2-overexpressing transgenic mice

were shown to be resistant to UVB-induced apoptosis (10). Furthermore, Bcl-2 knockout mice skin showed increased induction of apoptosis by UVB irradiation (11). The present study is in line with these results and using adenovirus vector we demonstrated for the first time the suppressive effect of Bcl-2 against apoptotic cell induction both *in vitro* and *in vivo*.

The effect of Bcl-2 against apoptosis is influenced by Bax. Bcl-2 homodimer reveals antiapoptotic effect, while the effect is inhibited by Bax because of heterodimer complex formation of Bcl-2 and Bax protein. Thus the ratio of these proteins is critical for determining cell death or cell survival (4). UVB irradiation has been known to decrease Bcl-2 expression without alteration of Bax in rat epidermis (12). In the present study, Bax expression level was not altered in bcl-2 transfected NMK cells, suggesting that the increased Bcl-2 is responsible for the suppressive effect against the UVB-induced apoptosis. Recently, Cho et al report that the keratinocytes derived from Bax-deficient mice showed resistance to UVB-induced apoptosis, showing the important role of Bcl-2 against UVB-induced apoptosis (13).

Various gene transfer systems have been described in keratinocytes. These include retrovirus vector system (14, 15), DNA-liposome method (16), and direct injection of naked DNA (17). Using NMK cells, we have tried liposomal transfection , but the efficacy was below 5% (data not shown). Further, it has been unsuccessful to establish gene-integrated cells using primary cultured keratinocytes. In this study, we showed that adenovirus vector quite efficiently (albeit transiently) introduced bcl-2 into primary cultured keratinocytes. Although relatively high concentrations of virus particles were required, adenovirus vector was shown to be remarkably useful for gene transfer into tape-stripped mice skin *in vivo*. These results are consistent with those of Lu et al, who successfully transfected lac*Z* gene using adenovirus vector through tape-stripped epidermis (8).

In conclusion, our results indicate that Bcl-2 markedly suppresses UVB-induced apoptosis that is accompanied with inhibition of caspase 9 and 3 in keratinocytes. We also showed that adenovirus vector is an efficient gene delivery system into keratinocytes both *in vitro* and *in vivo*.

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