UVB-induced anti-survival and pro-apoptotic effects on HaCaT human keratinocytes via caspase- and PKC-dependent downregulation of PKB, HIAP-1, Mcl-1, XIAP and ER stress

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Received September 4, 2013; Accepted December 12, 2013

DOI: 10.3892/ijmm.2013.1595

Abstract. Evidence suggests that solar ultraviolet B (UVB) radiation inhibits growth and/or induces apoptosis of human skin cells. However, mechanisms underlying the UVB-induced anti-survival and pro-apoptotic effects on human skin cells remain unclear. In this study, we investigated the effect of UVB radiation on survival and apoptosis of HaCaT human keratinocytes and determined possible molecular, cellular and signaling mechanisms including cross-regulation, which are responsible for the UVB's anti-survival and/or pro-apoptotic effects. The results showed that UVB radiation at 400 mJ/cm^2 for 8 h largely decreased cell survival and induced DNA fragmentation, an index of apoptosis, in HaCaT human keratinocytes. On a mechanistic level, UVB radiation triggered the activation of caspase-9, cleavage of poly(ADP-ribose) polymerase, and downregulation of myeloid cell leukemia-1 (Mcl-1), human inhibitor of apoptosis protein-1 (HIAP-1), X-linked IAP (XIAP), and protein kinase B (PKB), but did not affect the expression of B-cell lymphoma-2 in HaCaT cells. UVB radiation also upregulated the expression of glucose-regulated protein 78 (GRP78), an endoplasmic reticulum (ER) stress marker, in HaCaT cells. Of note, results of pharmacological inhibition studies have demonstrated that pretreatment with z-VAD-fmk, a pan-caspase inhibitor strongly attenuated UVB-induced apoptosis, the activation of caspase-9, downregulation of Mcl-1, XIAP and PKB (but not HIAP-1), and upregulation of GRP78, while pretreatment with GF109203 or GO6983, pan-PKC inhibitors, substantially blocked the UVB-induced reduction of cell survival, activation of caspase-9, downregulation of HIAP-1, XIAP, and PKB (but not Mcl-1), and GRP78 upregulation in HaCaT cells. Collectively, these results demonstrated that UVB has strong anti-survival and pro-apoptotic effects on HaCaT cells and the effects were largely mediated via the activation of caspase-9 and protein kinase Cs, which subsequently downregulated PKB, XIAP, HIAP-1 and Mcl-1, and triggered ER stress.

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Key words: ultraviolet B, caspase-9, protein kinase Cs, myeloid cell leukemia-1, inhibitor of apoptosis proteins, protein kinase B, HaCaT

Introduction

Solar ultraviolet (UV) radiation is considered a major source of environmental damage for human skin (1). Results of previoius studies have shown that exposure of UV radiation in skin cells triggers various cell changes, including generation of reactive oxidative stress, cell cycle arrest, and altered expression of genes encoding inflammation-related proteins (2-4). Strong evidence suggests that UV induces cell membrane disruption as well as nuclear DNA damage, resulting in skin cell loss and/or apoptosis (5-8). It is assumed that keratinocytes are the most numerous cells in human skin and likely the first cells to be damaged by UV radiation (9). However, the molecular and cellular mechanisms underlying UV-induced apoptosis in keratinocytes remain to be adequately determined.

Mounting evidence suggests that the induction of keratinocyte apoptosis by UVB radiation is mediated by the action of a variety of proteins and/or factors. For example, it has been previously shown that activation of caspase-9, a member of the caspase family, is important in initiating apoptosis in keratinocytes exposed to UVB radiation (10). Moreover, it has been demonstrated that several members of the B-cell lymphoma-2 (Bcl-2) family, such as Bcl-2 and myeloid cell leukemia-1 (Mcl-1), are involved in UV-induced apoptosis in keratinocytes (11-14). Involvement of the downregulation of certain members of the inhibitor of apoptosis protein (IAP) family, including human IAP-1 (HIAP-1), HIAP-2, X-linked IAP (XIAP), and survivin, in the UVB-induced apoptosis of keratinocyte has also been proposed (15-17). A number of studies have emphasized that activities of multiple signaling proteins, such as protein kinase B (PKB), mitogen-activated protein kinases (MAPKs), and protein kinase Cs (PKCs), are necessary for UVB-induced apoptosis of human keratinocytes and skin cell damage (18-21). It has also been shown that forcible overexpression of C/EBP homologous protein (CHOP), a transcription factor induced by endoplasmic reticulum (ER) stress,

causes apoptosis in keratinocytes in culture (22), suggesting that ER stress may play a role in UVB-induced keratinocyte apoptosis (22). However, the role and/or cross-regulation (crosstalk) among the abovementioned anti-apoptotic and signaling proteins and ER stress pathways in UVB-induced growth inhibition and/or apoptosis of keratinocytes remain to be determined.

In this study, we investigated the effect of UVB on survival and apoptosis of HaCaT human keratinocytes and determined possible molecular, cellular and signaling mechanisms including cross-regulation, which are responsible for the UVB's anti-survival and/or pro-apoptotic effects.

Materials and methods

Materials. DMEM/F12, penicillin-streptomycin, and fetal bovine serum (FBS) were purchased from WelGENE (Daegu, Korea). Enzyme-linked chemiluminescence (ECL) western detection reagents were purchased from Thermo Scientific (Waltham, MA, USA). Bradford reagent was purchased from Bio-Rad (Hercules, CA, USA). Antibodies of procaspase-9 and -3 were purchased from Stressgen Biotechnologies (Victoria, BC, Canada). Antibodies of Mcl-1, Bcl-2 and glucose-regulated protein 78 (GRP78) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies of XIAP and HIAP-1 were purchased from R&D Systems (Minneapolis, MN, USA). An antibody of poly(ADP-ribose) polymerase (PARP) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Antibodies of phospho-PKB (p-PKB) and total PKB (T-PKB) were purchased from Cell Signaling Technology (Danvers, MA, USA). N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk), GF109203X, GO6983, and proteinase inhibitor cocktail (100X) were purchased from Calbiochem (Madison, WI, USA). Plasticwares, including 6- and 24-well plates, were purchased from SPL Life Sciences Co., Ltd. (Gyeonggi-do, Korea). Other reagents, including mouse monoclonal anti-human actin antibody, were purchased from Sigma (St. Louis, MO, USA).

Cell culture. HaCaT human keratinocytes [Korean Cell Line Bank (KCLB), Seoul, Korea] were grown at 37˚C in a humidified condition of 95% air and 5% CO_2 in DMEM/F12 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

UVB radiation. UVB was supplied by a closely spaced array of seven Westinghouse FS-40 sunlamps, which delivered uniform radiation at a distance of 38 cm. The energy output of UVB (290-320 nm) at 38 cm was measured with a UVB photometer (IL1350 photometer; International Light, Newburyport, MA, USA). Briefly, for the preparation of whole cell lysate mentioned below, HaCaT cells were seeded in a 60-mm culture dish at a density of $1.6x10^6$ cells in 4 ml volume the day prior to UVB radiation. To prevent light absorption by cell culture medium, the medium was removed from just prior to UVB radiation and replaced with a thin layer of phosphate-buffered saline (PBS) to cover cells. Cells were then exposed for 0, 50, 100, 200 and 400 sec of UVB, corresponding to the dose of 0, 50, 100, 200 and 400 mJ/cm^2, respectively. PBS was then removed from cells after the specified time of UVB radiation. Fresh culture medium was added to cells exposed to UVB radiation for an additional 8 h.

Preparation of whole cell lysates. HaCaT cells ($1.6x10^6$/4 ml) plated in a 60-mm culture dish overnight were initially pretreated with or without a broad-spectrum caspase inhibitor (z-VAD-fmk) or a pan-PKC inhibitor (GF109203X or GO6983) for 1 h and then treated with or without UVB at 400 mJ/cm^2 for an additional 8 h. The conditioned cells were then washed twice with PBS and exposed to a lysis buffer [50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.25% sodium deoxycholate, 1% Triton X-100, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, proteinase inhibitor cocktail (1X)]. Whole cell lysates were collected in a 1.5 ml tube and centrifuged for 20 min at 4˚C at 14,240 x g. The supernatant was removed and its protein concentration was determined with Bradford reagent.

Western blotting. Whole cell lysates (50 μg protein) were separated by SDS-PAGE (10%) and transferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA). The membrane was washed with TBS (10 mM Tris, 150 mM NaCl) supplemented with 0.05% (vol/vol) Tween-20 (TBST) followed by blocking with TBST containing 5% (wt/vol) non-fat dry milk. The membrane was incubated with respective antibody specific for procaspase-9 (1:2,000), procaspase-3 (1:2,000), PARP (1:2,000), PKB (1:2,000), HIAP-1 (1:2,000), XIAP (1:1,000), Mcl-1 (1:1,000), Bcl-2 (1:1,000), GRP78 (1:1,000) or actin (1:5,000) at 4˚C overnight. The membrane was then exposed to secondary antibodies coupled with horseradish peroxidase for 2 h at room temperature. The membrane was washed three times with TBST at room temperature. Immunoreactivities were detected by ECL reagents. Equal protein loading was assessed by expression levels of actin protein.

Cell count assay. HaCaT cells were seeded in 24-well plates at a density of $2x10^5$ cells in 500 μl volume overnight. The cells were then exposed to UVB (400 mJ/cm^2) for 2, 5 or 8 h. The number of surviving YD-8 cells that could not be stained with trypan blue dye was counted under microscope. Approximately <100 cells were counted for the analysis.

Measurement of DNA fragmentation. HaCaT cells ($1.6x10^6$/4 ml) were seeded in a 60-mm culture dish the day prior to UVB radiation. The cells were incubated with varying doses of UVB for 8 h, harvested, washed, and lysed in a buffer [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 then added and the cells were incubated at 55˚C for 18 h. The lysates were centrifuged at 10,000 x g for 20 min. Genomic DNA in the supernatant was extracted with equal volume of neutral phenol-chloroform-isoamyl alcohol mixture (25:24:1), and analyzed by electrophoresis on 1.7% agarose gel. DNA was visualized and photographed under UV illumination after staining with ethidium bromide (0.1 μg/ml).

Statistical analysis. Cell count analysis was performed in triplicate and repeated three times. Data were expressed as mean ± standard error (SE). Significant differences were

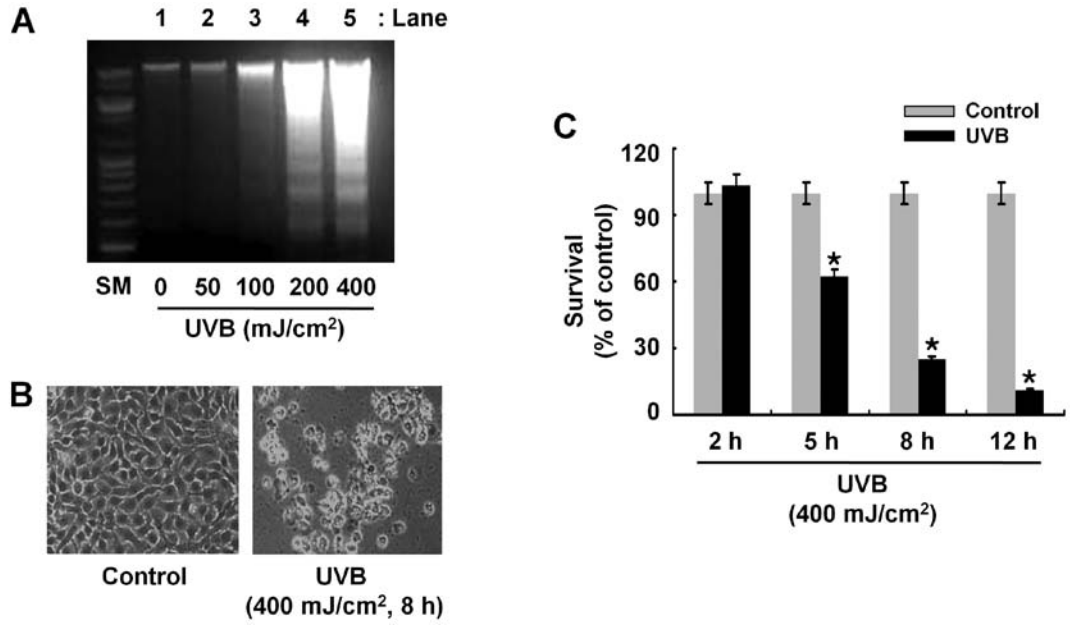

Figure 1. The effect of ultraviolet B (UVB) radiation on the survival and apoptosis of HaCaT cells. (A) HaCaT cells were treated with or without UVB at the indicated doses for 8 h. Extra-nuclear fragmented DNA from the conditioned cells was then extracted, and analyzed on a 1.7% agarose gel. Results are representative of three independent experiments. SM, DNA size marker. (B) HaCaT cells were treated with or without UVB (400 mJ/cm^2) for 8 h. Morphological change was then observed by microscopy. Results are representative of three independent experiments. (C) HaCaT cells were treated with or without UVB (400 mJ/cm^2) at the indicated times. At each time point, the number of surviving HaCaT cells was counted under a microscope by cell count analysis. Data are the mean ± SE of three independent experiments. The number of surviving cells was normalized as the percentage of UVB-free control. *P<0.05 compared to the value obtained at the same time-point (h) in the absence of UVB exposure.

determined by one-way ANOVA. P<0.05 was considered to indicate statistical significance.

Results

UVB radiation leads to reduction of cell survival and induction of apoptosis of HaCaT cells. Initially, we investigated whether UVB radiation induces apoptosis of HaCaT cells. In this study, apoptosis induction by UVB was assessed by the ability of UVB to induce genomic DNA fragmentation, an index of apoptosis in HaCaT cells. There was no DNA fragmentation in HaCaT cells exposed to UVB radiation at 50 or 100 mJ/cm^2 for 8 h (lanes 2 or 3) (Fig. 1A). However, there was strong induction of DNA fragmentation in HaCaT cells exposed to UVB at 200 or 400 mJ/cm^2 (lanes 4 or 5). Cells undergoing apoptosis have distinct morphological changes, such as cell shrinkage. Compared with the control, UVB (400 mJ/cm^2, 8 h) radiation induced severe cell shrinkage of HaCaT cells, supporting the apoptosis-inducing effects of UVB on HaCaT cells (Fig. 1B). Using 400 mJ/cm^2, time course experiments were conducted to determine the effect of UVB (400 mJ/cm^2) radiation on survival of HaCaT cells over time. Data of cell count analysis demonstrated ~47, 73 and 90% reduction of HaCaT cell survival by UVB radiation at 5, 8 and 12 h, respectively (Fig. 1C). These results collectively suggest that UVB radiation at 400 mJ/cm^2 for 8 h has strong anti-survival and pro-apoptotic effects on HaCaT cells.

UVB radiation leads to activation of caspase-9 and -3 and cleavage of PARP in HaCaT cells. Induction of apoptosis is influenced by the activity of a number of proteins, including caspases. The effect of UVB radiation on the activation of caspases in HaCaT cells was determined using western blot analysis. Activation of caspases by UVB was assessed by the ability of UVB to alter the cellular levels of the inactive proform and active form (cleaved product) of caspases in HaCaT cells. Compared with the control (lane 1), UVB radiation at 50 or 100 mJ/cm^2 for 8 h did not affect the cellular levels of procaspase-9 and -3 in HaCaT cells (lanes 2 or 3) (Fig. 2). However, the same time exposure of UVB at 200 or 400 mJ/cm^2 decreased the cellular levels of procaspase-9 and -3, while largely increasing those of cleaved caspase-9 and -3. PARP is a downstream substrate of caspase-9 and -3. There was a UVB radiation dose-dependent increase in the cellular levels of cleaved PARP in HaCaT cells (lanes 2-5) in which UVB at 400 mJ/cm^2 induced the highest cellular accumulation of cleaved PARP, confirming the UVB-induced activation of caspase-9 and -3 in HaCaT cells.

UVB radiation leads to the altered expression of Mcl-1, HIAP-1, XIAP and PKB in HaCaT cells. We investigated whether UVB radiation affects expression of anti-apoptotic and signaling proteins involved in cell survival and/or apoptosis, including the Bcl-2 or IAP family and PKB, in HaCaT cells. Compared with the control (lane 1), there was little effect on Mcl-1 protein expression by UVB at 50, 100 or 200 mJ/cm^2 for 8 h (lanes 2-4) (Fig. 3). However, there was a strong downregulation of Mcl-1 protein expression by UVB at 400 mJ/cm^2. UVB radiation at the doses applied did not affect Mcl-1 protein expression in HaCaT cells (lanes 2-5) compared with the control (lane 1). Instead, a slight increase of Bcl-2 protein expression by UVB at 200 or 400 mJ/cm^2 was observed. UVB at 50 or 100 mJ/cm^2 did not affect HIAP-1 and XIAP protein expression (lanes 2 or 3), but there was strong

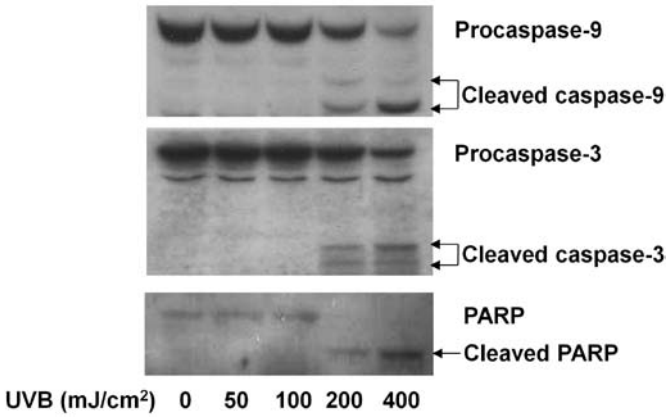

Figure 2. The effect of ultraviolet B (UVB) radiation on the activation of caspases in HaCaT cells. HaCaT cells were treated with or without UVB at the indicated doses for 8 h. Whole cell lysates were prepared, and analyzed by western blotting using specific antibody of procaspase-9, -3 or poly(ADP-ribose) polymerase (PARP). Results are representative of three independent experiments.

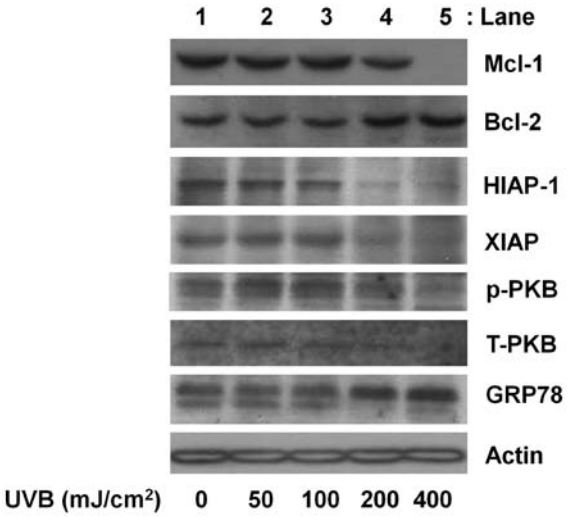

Figure 3. The effect of UVB radiation on the expression and/or phosphorylation of myeloid cell leukemia-1 (Mcl-1), human inhibitor of apoptosis protein-1 (HIAP-1), B-cell lymphoma-2 (Bcl-2), HIAP-1, X-linked IAP (XIAP), protein kinase B (PKB) and glucose-regulated protein 78 (GRP78) in HaCaT cells. HaCaT cells were treated with or without UVB at the indicated doses for 8 h. Whole cell lysates were prepared, and analyzed by western blotting using the specific antibodies of Mcl-1, HIAP-1, Bcl-2, HIAP-1, XIAP, PKB and GRP78. p-PKB, phosphorylated PKB; T-PKB, total PKB. Results are representative of three independent experiments.

repression of HIAP-1 and XIAP proteins by UVB at 200 or 400 mJ/cm^2 (lanes 4 or 5). In the case of PKB, UVB at 50, 100 or 200 mJ/cm^2 did not affect PKB protein phosphorylation and expression (lanes 2-4) compared with the control (lane 1). However, phosphorylated levels of PKB and total expression levels of this protein by UVB at 400 mJ/cm^2 (lane 5) were markedly reduced. Evidence suggests that ER stress plays a role in the induction of apoptosis. Cells with ER stress express high GRP78, a molecular chaperone protein that involves in protein folding. Compared with the control (lane 1), no enhancement of GRP78 protein expression in HaCaT cells exposed to UVB at 50 or 100 mJ/cm^2 (lanes 2 or 3) was identified. However, a marked increase in GRP78 protein expression by UVB at 200 or 400 mJ/cm^2 (lanes 4 or 5) was noted. Actin protein expression of the control was not altered by UVB at the doses tested.

Important roles of the caspase activation in UVB-induced apoptosis and downregulation of Mcl-1, XIAP and PKB (but not HIAP-1) in HaCaT cells. Involvement of caspases in the UVB-induced apoptosis of HaCaT cells was determined using z-VAD-fmk, a broad-spectrum caspase inhibitor. The UVB-induced apoptosis in HaCaT cells (lane 2) was strongly blunted by the caspase inhibitor (lane 3) (Fig. 4A). The UVB-induced morphological changes (cell shrinkage) of HaCaT cells were also largely blocked by z-VAD-fmk (Fig. 4B). Notably, the UVB-induced activation of caspase-9 and production of cleaved PARP in HaCaT cells (lane 2) was not shown in the presence of z-VAD-fmk (lane 3), suggesting the drug efficacy to inhibit the caspase pathway (Fig. 4C). Of note, the UVB-induced downregulation of Mcl-1 and XIAP, but not HIAP-1, in HaCaT cells (lane 2) was strongly blocked by z-VAD-fmk (lane 3). Furthermore, the UVB-induced downregulation of phosphorylated and total PKB proteins in HaCaT cells (lane 2) was largely inhibited by z-VAD-fmk (lane 3). Actin protein expression remained unchanged under these experimental conditions (Fig. 4C).

Involvement of PKCs in the UVB-induced reduction of cell survival and downregulation of HIAP-1, XIAP, and PKB (but not Mcl-1) in HaCaT cells. PKCs are involved in cell survival and/or apoptosis of keratinocytes exposed to UVB. Using GF109203X or GO6983, a broad-spectrum of PKC inhibitor, we determined whether activities of PKCs are linked to the UVB-induced anti-survival and/or pro-apoptotic responses in HaCaT cells. The anti-survival effect of UVB on HaCaT cells (column 2) was largely blocked in the presence of GF109203X (column 3) or GO6983 (column 4) (Fig. 5A). Notably, the UVB effects on activation of caspase-9, cleavage of PARP, downregulation of HIAP-1, XIAP and PKB in HaCaT cells (lane 2) was partially or largely blunted by GF109203X (lane 3) or GO6983 (lane 4) (Fig. 5B). The UVB-induced downregulation of Mcl-1 (lane 2) was not changed by each PKC inhibitor (lanes 3 or 4). Actin protein expression remained constant by treatment with or without UVB in the absence or presence of GF109203X or GO6983 (Fig. 5B).

Discussion

Solar UVB radiation induces various types of skin damage, some of which may lead to loss of keratinocytes and/or induction of keratinocyte apoptosis. In the present study, we have demonstrated the ability of UVB radiation at 400 mJ/cm^2 for 8 h to strongly reduce cell survival and induce apoptosis of HaCaT human keratinocytes. Our data indicate that the UVB-induced anti-survival and pro-apoptotic effects on HaCaT cells are mediated through modulation of the expression and/or activity of caspases, PKCs, PKB, XIAP, HIAP-1 and/or Mcl-1, along with induction of ER stress.

Accumulating evidence suggests that UVB radiation, depending on its dose and time, leads to a decrease in cell survival and/or induction of apoptosis in various skin cells,

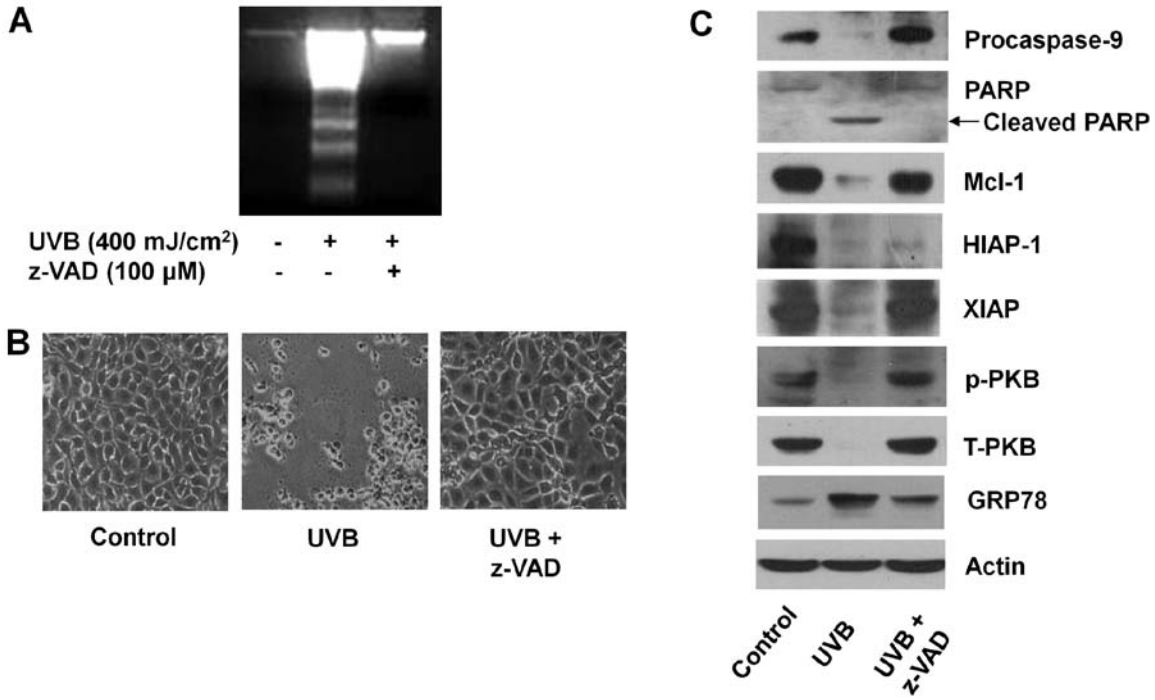

Figure 4. The effect of ultraviolet B (UVB) radiation and/or z-VAD-fmk on the survival, apoptosis, and expression of myeloid cell leukemia-1 (Mcl-1), human inhibitor of apoptosis protein-1 (HIAP-1), B-cell lymphoma-2 (Bcl-2), HIAP-1, X-linked IAP (XIAP), protein kinase B (PKB), and glucose-regulated protein 78 (GRP78) in HaCaT cells. (A) HaCaT cells were pretreated with or without z-VAD-fmk, a pan-caspase inhibitor for 1 h and then treated with or without UVB in the absence or presence of the caspase inhibitor for 8 h. Extra nuclear-fragmented DNA from the conditioned cells was then extracted, and analyzed on a 1.7% agarose gel. (B) HaCaT cells were pretreated with or without z-VAD-fmk for 1 h and then treated with or without UVB in the absence or presence of the caspase inhibitor for 8 h. Morphological change was then observed via microscopy. (C) HaCaT cells were pretreated with or without z-VAD-fmk for 1 h and then treated with or without UVB in the absence or presence of the caspase inhibitor for 8 h. Whole cell lysates were prepared, and analyzed by western blotting using specific antibodies of procasapse-9, poly(ADP-ribose) polymerase (PARP), Mcl-1, HIAP-1, XIAP, PKB and GRP78. p-PKB, phosphorylated PKB; T-PKB, total PKB. Results are representative of three independent experiments.

including normal keratinocytes, HaCaT cells, and JB6 murine epidermal cells (21,26,27). Cells undergoing apoptosis have distinct biochemical and morphological characteristics, such as nuclear DNA fragmentation and cell shrinkage (28). Bearing this in mind and considering the present findings that UVB radiation induces nuclear DNA fragmentation and cell shrinkage in HaCaT cells (Fig. 1A and B), it appears that UVB induces HaCaT cell death by apoptosis.

Induction of apoptosis is mainly linked to the intrinsic (mitochondrial) and extrinsic (death receptor-mediated) pathways. Caspases, a group of the essential proteases required for the execution of cell death by apoptotic stimuli (29), are key to both apoptotic pathways. Caspases are synthesized as zymogens (inactive precursors) in resting cells, however, following exposure to apoptotic stimuli, they become processed via partial proteolytic cleavage and are activated in cells. Active caspases then cleave many cellular proteins, including PARP and other vital proteins, leading to the induction and/or execution of apoptosis (29-31). Among caspases, caspase-9 and -8 is an initiator caspase of the extrinsic and intrinsic apoptotic pathways, respectively (29,32). Findings of previous studies have shown that UVB radiation triggers the activation of the intrinsic and extrinsic pathways (9-11,33). However, evidence suggests that the UVB-induced apoptosis of human keratinocytes is primarily mediated via the intrinsic pathway (15,34). Consistent with those findings, the present study has also demonstrated the UVB-induced activation of caspase-9 (Fig. 2), which is crucial in the UVB-induced apoptosis of HaCaT cells (Fig. 4A).

Members of the Bcl-2 family, including Bcl-2 and Mcl-1, are anti-apoptotic proteins and participate in apoptosis initiation and/or caspase activation by regulating the mitochondrial membrane integrity (23,24). Earlier studies have demonstrated that Bcl-2 overexpression abrogates the UVB-induced apoptosis in human keratinocytes *in vitro* and *in vivo* (6,11). Additionally, Mcl-1 is a major epithermal survival protein and alteration of Mcl-1 expression influences the UV-induced apoptosis in normal keratinocytes (14), suggesting involvement of Bcl-2 and Mcl-1 in the UV-induced apoptosis of keratinocyes. In this study, however, we have observed that UVB radiation strongly downregulates the expression of Mcl-1, but does not affect that of Bcl-2 in HaCaT cells (Fig. 3). Thus, it is assumed that downregulation of Mcl-1, but not Bcl-2, may contribute to the UVB-induced apoptosis, reduction of cell survival, and/or activation of caspase-9 in HaCaT cells. Differences in the expression levels of Bcl-2 protein in HaCaT cells exposed to UVB in this study and previous ones may be due to different experimental conditions (UVB radiation dose and exposure time) applied.

The members of IAPs, including HIAP-1, HIAP-2, XIAP and survivin, are other suppressors of apoptosis (25) and inhibitors of caspases (35,36). In particular, XIAP is shown to directly inhibit caspase-9 and -3 (36). Studies have recently suggested that UVB radiation decreases the cellular levels of

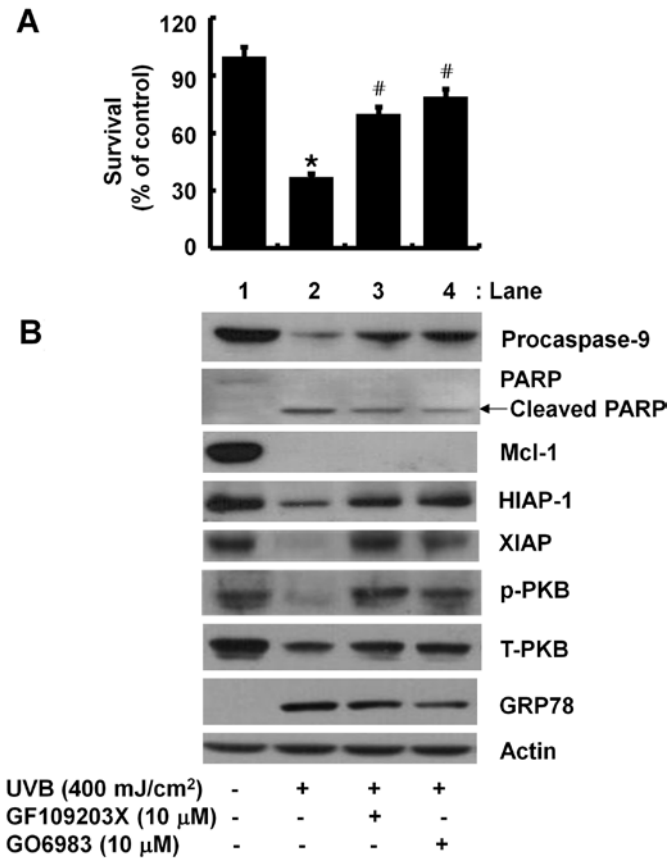

Figure 5. The effect of ultraviolet B (UVB) radiation and/or GF109203X or GO6983 on the survival and expression of myeloid cell leukemia-1 (Mcl-1), human inhibitor of apoptosis protein-1 (HIAP-1), B-cell lymphoma-2 (Bcl-2), HIAP-1, X-linked IAP (XIAP), protein kinase B (PKB), and glucose-regulated protein 78 (GRP78) in HaCaT cells. (A) HaCaT cells were pretreated with or without GF109203X or GO6983, a broad spectrum PKC inhibitor for 1 h and then treated with or without UVB in the absence or presence of the PKC inhibitor for 8 h. Data are the mean ± SE of three independent experiments. The number of surviving cells was normalized as a percentage of UVB-free control. *P<0.05 compared to the value of UVB-free control. #P<0.05 compared to the value obtained at UVB treatment without any drug. (B) HaCaT cells were pretreated without or with GF109203X or GO6983 for 1 h and then treated with or without UVB in the absence or presence of the PKC inhibitor for 8 h. Whole cell lysates were prepared, and analyzed by western blotting using specific antibodies of procasapse-9, poly(ADP-ribose) polymerase (PARP), Mcl-1, HIAP-1, XIAP, PKB and GRP78. p-PKB, phosphorylated PKB; T-PKB, total PKB. Results are representative of three independent experiments.

HIAP-1, HIAP-2, survivin, and livin in normal human keratinocytes (15) and of XIAP in HaCaT cells (16). In this study, we have also demonstrated that UVB radiation leads to the downregulation of HIAP-1 and XIAP in HaCaT cells (Fig. 3), which suggests that downregulation of HIAP-1 and XIAP may facilitate the UVB-induced apoptosis and activation of caspase-9 in HaCaT cells. At present, we have limited knowledge regarding the mechanisms underlying the UVB-induced downregulation of Mcl-1, HIAP-1 and XIAP in HaCaT cells. Notably, the present findings with large blockage of the UVB-induced downregulation of Mcl-1 and XIAP, but not HIAP-1, in HaCaT cells by z-VAD-fmk, a pan-caspase inhibitor (Fig. 4C) indicate that caspases are responsible for the UVB-induced Mcl-1 and XIAP downregulation (proteolysis) in HaCaT cells.

PKB is a protein kinase involved in cell survival and/or the apoptosis of a variety of cells. Notably, it has been shown that UVB radiation induces the rapid activation of PKB (within 15 min), which contributes to the UVB-activated cell survival pathway in human skin *in vivo* (37). Moreover, a protective role of PKB in the early-activated apoptotic pathway in keratinocytes exposed to UVB radiation via BAD phosphorylation and translocation has been suggested (38). In this study, we have demonstrated that UVB radiation downregulates phosphorylated and total PKB proteins in HaCaT cells (Fig. 3) and importantly the downregulation is largely blocked by the pan-caspase inhibitor z-VAD-fmk (Fig. 4C). These results suggest that elimination of PKB further contributes to the UVB-induced reduction of cell survival and/or apoptosis of HaCaT cells and that caspases are also responsible for the UVB-induced downregulation of PKB in the cells.

PKCs are a group of serine/threonine kinases involved in cell proliferation, survival and apoptosis (39,40). At present, 12 members of PKCs have been identified, and can be classified into three groups based on the co-factors required for activation, including the Ca^{2+} and DAG-dependent classical PKCs (PKC-α, -β1, -β2 and -γ), the DAG-dependent, Ca^{2+}-independent novel PKCs (PKC-δ, -η, -ε and -θ), and the DAG- and Ca^{2+}-independent atypical PKCs (PKC-λ and -ζ) (41). Notably, results of previous studies suggest a link between the activity of PKCs and keratinocyte survival and/or apoptosis in response to UVB radiation. For instance, UVB radiation causes the activation of PKC-δ, ε, ζ, λ and η, leading to apoptosis or cell survival (21,42). It has also been reported that PKC-δ is activated in a caspase-dependent manner, and this activation of PKC-δ contributes to the UVB-induced apoptosis in human keratinocytes (18,20,43). However, PKC-ζ is suggested to be rather anti-apoptotic in response to UV (44). Notably, results of the present study, albeit activation of PKCs by UVB radiation in HaCaT cells is not directly measured, demonstrate that GF109203X or GO6983, pan-PKC inhibitors, effectively blocks the UVB-induced reduction of cell survival (Fig. 5A), activation of caspase-9, and downregulation of HIAP-1, XIAP and PKB in HaCaT cells (Fig. 5B), which strongly suggest a necessity for PKCs activities in such UVB-induced growth inhibition and cellular changes in HaCaT cells. Given that GF109203X or GO6983 is a pan-PKC inhibitor, it is likely that multiple PKC members may be involved in UVB-induced growth inhibition and cellular responses in HaCaT cells. Future studies, using siRNA and/or cDNA transfection targeting each PKC, are required to differentiate which isotype of PKCs is responsible for the processes.

Evidence suggests that ER stress also mediates induction of apoptosis. Cells undergoing ER stress have distinct characteristics, including accumulation of unfolded and misfolded proteins, upregulation of molecular chaperones (e.g., GRP78) and transcription factors (e.g., CHOP), phosphorylation of eukaryotic translation initiation factor 2α and inhibition of global translation, and reduction of p90 activation of transcription factor 6 (45-48). In a recent study, it was shown that UVB radiation at low dose (10 mJ/cm^2) for 4 h induces ER stress, as assessed by the accumulation of unfolded proteins in ER, CHOP upregulation, and downregulation of ATF6, in HaCaT cells, without growth retardation (49). However, the same group has also demonstrated that under this UVB radiation, unfolded protein

response and ER-associated degradation systems are activated in order to protect cells from UVB-induced ER stress in HaCaT cells. In the present study, we have demonstrated that UVB radiation (400 mJ/cm^2, 8 h) enhances the expression of GRP78 in HaCaT cells (Fig. 3), confirming UVB-induced ER stress in HaCaT cells. Currently, the significance or role of the induced ER stress in HaCaT cells exposed to UVB radiation remains to be clarified. However, considering the present findings that UVB radiation (400 mJ/cm^2, 8 h) strongly reduces cell survival and induces apoptosis of HaCaT cells, whether the induced ER stress may facilitate and/or contribute to the UVB-induced apoptotic responses in HaCaT cells remains to be determined. Little is known about crosstalk between PKCs or caspases and ER stress in cells, including keratinocytes, in response to UVB radiation. Findings of the present study have shown that the pan-caspase inhibitor (z-VAD-fmk) or pan-PKC inhibitor (GO6983) substantially interferes with the UVB-induced enhancement of GRP78 expression in HaCaT cells (Fig. 5A), suggesting a partial crosstalk between caspases or PKCs and ER stress in the HaCaT cells exposed to UVB radiation.

In summary, findings of this study have, to the best of our knowledge, demonstrated for the first time that UVB radiation has strong anti-survival and pro-apoptotic effects on HaCaT cells and the effects are mediated through the activation of caspase-9 and PKCs, which subsequently downregulates PKB, HIAP-1, Mcl-1 and XIAP, and triggers ER stress.

Acknowledgements

The authors would like to thank Ms. Sun-Young Cho for her excellent technical assistance.

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