Activation of p38 Mitogen-Activated Protein Kinase and Caspases in UVB-Induced Apoptosis of Human Keratinocyte HaCaT Cells

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Exposure of human keratinocyte HaCaT cells to ultraviolet B-irradiation induced apoptotic morphologic changes. In this study, we found that the ultraviolet B irradiation (0.25 J per cm^2) induced phosphorylation of p38 mitogen-activated protein kinase and c-jun N-terminal protein kinase, and also significant activation of caspase-3 (CPP32-like protease) and a small increase of caspase-1 (ICE-like protease) activity in the early stages of ultraviolet B-induced apoptosis. Pretreatments of the cells with a p38 mitogen-activated protein kinase inhibitor, SB203580, and a caspase-3 inhibitor, Ac-Asp-Met-Gln-Asp-1-aldehyde, suppressed the ultraviolet B irradiation-induced apoptosis by ≈60% as estimated by nuclear staining and DNA laddering. Pretreatment with caspase-1 inhibitor, Ac-Tyr-Val-Lys-Asp-aldehyde was without effect. Ultraviolet B-induced caspase-3 activation resulted in cleavage of poly(ADP) ribose polymerase, which was abolished by the caspase-3 inhibitor. SB203580 pretreatment prevented activation of caspase-3 and caspase-1, and also suppressed the cleavage of poly(ADP) ribose polymerase. Neither ceramide generation nor sphingomyelinase activation (neutral and acid) was observed in the ultraviolet B-irradiated HaCaT cells. Also various antioxidants did not affect the caspase activation induced by ultraviolet B irradiation. These results indicated that activation of p38 mitogen-activated protein kinase upstream of caspases may play an important part in the apoptotic process of keratinocytes exposed to ultraviolet B irradiation. ***Keywords: apoptosis/caspase/p38 mitogen-activated protein kinase/ultraviolet B. J Invest Dermatol 112:769–774, 1999***

Keratinocytes cover the body surface which is exposed to various environmental factors such as ultraviolet (UV) and temperature. It is also well documented that UVB (290–320 nm) irradiation is hazardous to epidermis leading to sunburn characterized by erythema and edema as a result of increased vascular permeability. Despite abundant studies, however, the mechanisms underlying these biologic effects have not yet been elucidated fully. The effects of UV irradiation are frequently mediated by reactive oxygen species (ROS), and increased levels of hydrogen peroxides (H_2O_2), hydroxyl radicals, superoxide, and organic hydroperoxides are often observed in many different types of cells following UV exposure (Dixit *et al*, 1983; Pelle *et al*, 1990). Production of these ROS causes the programmed cell death (apoptosis) of keratinocytes (Stewart *et al*, 1996). Keratinocytes that are too severely damaged to repair the DNA induce apoptosis as the escape mechanism, thereby eliminating themselves in the interest of the rest of the organism. In this process, tumor suppresser gene p53 plays an important part (Ziegler *et al*, 1994). UV irradiation is known to arrest some cells during the G_1 phase of the cell cycle in a p53-dependent manner (Campbell *et al*, 1993). During this G_1 arrest some cells can repair DNA damage. Cells that do not have functional p53, however, cannot arrest in the G_1 phase after UV irradiation, and they are unable to repair the DNA damage, consequently leading to a higher incidence of p53 mutation.

Although recent studies have provided a lot of information regarding the prevention of skin carcinogenesis via apoptosis, limited investigations have been done for the signal transduction of keratinocyte apoptosis. Recent study has shown that UV irradiation induces activation of caspase-3, poly(ADP) ribose polymerase (PARP) cleavage, and finally apoptosis via the Fas/Fas ligand system in the human keratinocyte cell line HaCaT (Aragane *et al*, 1998). Fas has been reported to stimulate acidic sphingomyelinase (SMase) which hydrolyzes sphingomyelin to produce ceramide (Cifone *et al*, 1994; Tepper *et al*, 1995; Gulbins *et al*, 1995; Brenner *et al*, 1998), which is known to act as a potent inducer of apoptosis (Hannun, 1996; Yoshimura *et al*, 1998). Our previous study has shown that a cell-permeable ceramide (C2-ceramide) induced apoptosis in HaCaT cells (Iwasaki-Bessho *et al*, 1998). In this investigation we have examined the apoptotic signaling induced by UVB irradiation in HaCaT cells.

MATERIALS AND METHODS

Cell culture and UVB irradiation A HaCaT cell line was kindly supplied by Dr. T. Kuroki (University of Showa, Tokyo, Japan) with permission from Dr. N.E. Fusenig (German Cancer Research Center, Heidelberg, Germany, 1988). HaCaT cells were grown in Dulbecco's modified essential medium (Gibco-BRL, Grand Island, NY) supplemented

Manuscript received September 29, 1998; revised December 3, 1998; accepted for publication January 20, 1999.

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Abbreviations: JNK, c-jun N-terminal kinase; p38-MAPK, p38 mitogen activated protein kinase; ERK, extracellular signal-regulated kinase; PARP, poly(ADP) ribose polymerase; ROS, reactive oxygen species.

with 10% (vol/vol) fetal bovine serum (Irvine Scientific, Santa Ana, CA), 100 units penicillin per ml, and 100 μg streptomycin per ml in a humidified atmosphere containing 5% CO_2 at 37°C. Cells were passaged every 7 d at a 1:10 split. For the UV irradiation experiments, the cells (1×10^6) were grown in 100 mm culture dishes for 48 h. The cells were washed twice with phosphate-buffered saline (PBS) and exposed to UVB irradiation using BX-15 (ATTO, Tokyo, Japan), which emits most of their energy within the UVB range (290–320 nm) with an emission peak at 312 nm. The UV dose was measured using a ATV-3 W UV meter (ATTO, Tokyo, Japan). After irradiation, the cells were further incubated in the medium containing 5% fetal bovine serum albumin for different time periods and harvested by scraping. PBS-treated cells without UV irradiation served as the control.

Fluorescence microscopy Apoptotic cells induced by UVB irradiation were identified and quantitated by fluorescence microscope analysis with Hoechst 33258 staining (Shimizu *et al*, 1996). The harvested cells were stained with 10 μM Hoechst 33258 (bisbenzimide) (Wako, Osaka, Japan) for 10 min and examined under a fluorescence microscope (Olympus BX60) with excitation at 360 nm.

DNA fragmentation assay For DNA fragmentation assay, HaCaT cells were centrifuged at 3000 × *g* for 10 min, washed twice in PBS, and resuspended in ice-cold lysis buffer containing 10 mM Tris–HCl (pH 7.4), 10 mM EDTA-Na, 0.5% Triton X-100. The lysates were centrifuged at 16,000 × *g* for 20 min and the resulting supernatants were treated with proteinase K (20 mg per ml) (Boehringer, Mannheim, Germany) and DNase-free RNase (20 mg per ml) (Boehringer) for 1 h at 37°C. Genomic DNA was precipitated with ethanol and recovered by centrifugation at 16,000 × *g* for 15 min. The resulting pellets were dissolved in Tris–ethylediamine tetraacetic acid (EDTA) buffer and electrophoresed on 2.0% agarose gel. The DNA was stained with ethidium bromide (5 mg per ml) and visualized under UV light and photographed (ATTO, Tokyo, Japan).

Western blot analysis HaCaT cells were cultured in 1×10^6 cells per 100 mm dishes for 48 h at 37°C. Cells were harvested by scraping, and collected by centrifugation at 3000 × *g* for 10 min and washed twice with ice-cold PBS and then sonicated in 100 μl of ice-cold lysis buffer [1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium desoxycholate, 1 mM EDTA, 1 mM ethyleneglycol-1-bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 50 mM NaCl, 25 mM HEPES, 10 mM sodium fluoride, 1 mM sodium vanadate, 0.1 mM sodium molybdate, 1 mM phenylmethylsulfonyl fluoride, 10 μg leupeptin (Peptide Institute, Osaka, Japan) per ml, pH 7.4]. The extracted proteins (100–150 μg per lane) were separated by electrophoresis on 8 or 10% SDS–polyacrylamide gel and electrophoretically transferred on to polyvinylidene difluoride membranes. The membranes were probed with primary antibodies [anti-phospho-c-jun N-terminal kinase (JNK), anti-phospho-p38 mitogen activated protein kinase (p38-MAPK), and anti-phospho-extracellular signal-regulated-kinase (ERK) antibodies; New England Biolabs, Boston, MA and anti-PARP antibody; Santa Cruz, Biotechnology, Santa Cruz, CA] and then incubated with the anti-rabbit IgG horseradish peroxidase-coupled secondary antibody (Tago, Burlingame, CA). Detection was performed with the enhanced chemiluminescence (ECL) system (Amersham, Bucks., U.K.) according to the manufacturer's protocol.

Assay for caspase Cells were exposed to UVB irradiation and harvested by scraping. The collected cells (touched and detached) were centrifuged at 3000 × *g* for 10 min and washed two times with ice-cold PBS. Then the cells were sonicated in the buffer (100 μl) containing 20 mM HEPES, 0.1% β-octylglucoside, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 20 μg leupeptin per ml, pH 7.4. The lysate was spun down at 16,000 × *g* for 30 min, and the resulting supernatant (10 μg protein) was incubated in the presence of caspase-1 substrate, Tyr-Val-Ala-Asp-7-amino-4-trifluoromethyl Coumarin (YVAD-AFC) or caspase-3 substrate, carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl Coumarin (Z-DEVD-AFC) at 30°C for 30 min. Levels of released AFC were then measured using Versa Fluor Fluorometer (Bio-Rad, Tokyo Japan) with excitation at 390 nm and emission at 510 nm. Excitation and emission slit width were adjusted to 10 mm and 20 mm, respectively.

Measurement of ceramide production Cellular ceramide level was measured as previously described (Yoshimura *et al*, 1998). Briefly, extracted lipids were dried and incubated in 0.1 M KOH in chloroform/methanol (1:2, vol/vol) at 37°C for 1 h. Ceramide was converted to ceramide 1-[^{32}P]phosphate by *Escherichia coli* diacylglycerol kinase (Calbiochem, La Jolla, CA) in the presence of [γ-^{32}P]ATP (3000 Ci per mmol) (NEN Life Science Products, Boston, MA), and separated by high-performance thin-layer chromatography (HPTLC) (Whatman, Clifton, NJ) in chloroform/acetone/methanol/acetic acid/water (50:20:15:10:5, vol/vol). Following autoradiography, spots corresponding to ceramide 1-phosphate were scraped and the radioactivity was measured in a scintillation counter. Quantitation of ceramide was based on a standard curve of known amounts of ceramide. The changes in ceramide content were normalized based on total protein.

Assay for sphingomyelinase activity HaCaT cells were exposed to UVB irradiation and collected by centrifugation at 3000 × *g* for 10 min and washed two times with ice-cold PBS. Cells were then disrupted by sonication in buffer A (25 mM HEPES/NaOH buffer, pH 7.4, 100 mM KCl, 3 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg leupeptin per ml). After unbroken cells were removed by centrifugation at 900 × *g* for 5 min, the resulting supernatants were further centrifuged at 100,000 × *g* for 60 min. The precipitated membranes were washed once and resuspended in buffer A. The activities of both neutral and acid SMases were determined as previously described (Yoshimura *et al*, 1998). For the assay of magnesium-dependent neutral SMase, the membrane fractions (20 μg) were incubated with [methyl-^{14}C]sphingomyelin (NEN Life Science Products) (40,000 cpm in 1 nmol of bovine brain sphingomyelin (Sigma, St. Louis, MO) in 0.25% Triton X-100 solubilized by sonication) in 0.1 M Tris/HCl buffer (pH 7.4) containing 6 mM $MgCl_2$ for 1 h at 37°C. Then the reaction was stopped by the addition of 1.5 ml of chloroform/methanol (2:1, vol/vol) followed by 0.2 ml of H_2O. After phase separation, a portion of the upper phase was transferred to scintillation vials and the radioactivity was determined by liquid scintillation counting. Acid SMase activity was measured as above except that 0.1 M sodium acetate buffer, pH 5.0, containing 5 mM EDTA replaced the Tris/HCl buffer.

RESULTS

Nuclear staining and DNA laddering in HaCaT cells exposed to UVB irradiation Previous study has demonstrated that HaCaT cells exposed to UVB irradiation at a dose of 0.25 J per cm^2 induced significant apoptosis (Henseleit *et al*, 1996). In this study, we have examined the extent of apoptotic changes induced by UVB irradiation at various doses in HaCaT cells and observed that the marked apoptosis was induced at a dose of 0.25 J per cm^2. Subconfluent HaCaT cells were irradiated with UVB at a dose of 0.25 J per cm^2 and harvested at 12 h following irradiation. HaCaT cells were stained with Hoechst 33258 and observed under a fluorescence microscope. Nuclei of the control untreated HaCaT cells were stained uniformly with the dye, indicating that the nuclei were intact (**Fig 1*A***). Exposure of cells to UVB, however, resulted in nuclear fragmentation and condensation, which are characteristic of apoptotic changes (**Fig 1*B***). The extent of apoptosis induced by UVB irradiation was determined quantitatively by counting cells with fragmented and/or condensed nuclei. Approximately 40% of cells displayed apoptotic morphologies at 12 h after UVB irradiation. Preincubation with caspase-3 inhibitor, acetyl-Asp-Met-Gln-Asp-1-aldehyde (Ac-DMQD-CHO) (100 μM) or p38-MAPK inhibitor, SB203580 suppressed the UVB-induced nuclear fragmentation by ≈60% and 55%, respectively (**Fig 1*C***, ***D***). **Figure 2** showed DNA laddering in UVB-irradiated cells (8 h after irradiation). The caspase-3 inhibitor (Ac-DMQD-CHO) and p38-MAPK inhibitor (SB203580), but not caspase-1 inhibitor, acetyl-Tyr-Val-Lys-Asp-1-aldehyde (Ac-YVKD-CHO), reduced DNA laddering. These results suggested that caspase-3 and p38-MAPK may be involved in the apoptotic process of UVB-irradiated HaCaT cells.

Phosphorylation of mitogen-activated protein (MAP) kinase family induced by UVB irradiation We examined changes of activities of MAP kinase family, ERK, p38-MAPK, and JNK when HaCaT cells were exposed to UVB irradiation (0.25 J per cm^2). No significant phosphorylations of ERK1 and ERK2 were observed in UVB-irradiated HaCaT cells (**Fig 3*A***). On the other hand, phosphorylation of p38-MAPK was enhanced 3-fold at 2 h after UVB irradiation and was rapidly decreased thereafter (**Fig 3*B***). The rapid increase of phosphorylation of JNK1 (46 kDa) was also observed to occur peaking at 2 h following UVB irradiation. The

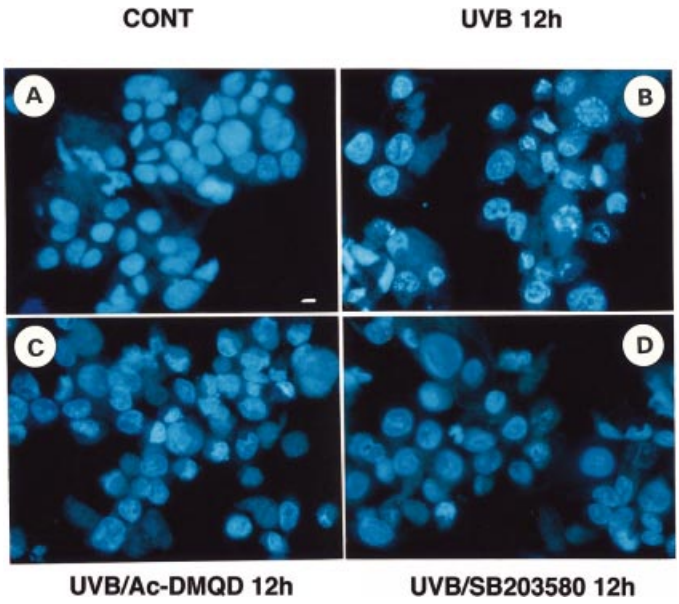

Figure 1. Temporal profiles of chromatin staining in HaCaT cells exposed to UVB irradiation. HaCaT cells were incubated for 12 h after untreated (*A*) or irradiated with UVB (0.25 J per cm^2) in the absence (*B*) or presence of caspase-3 inhibitor 100 μM Ac-DMQD (*C*) or p38-MAPK inhibitor 10 μM SB203580 (*D*). Nuclei were stained with Hoechst 33258 as described under *Materials and Methods*, and fluorescence photographs were taken under a fluorescent microscope. The photographs are representative of at least three different cultures. *Scale bar*: 10 μm.

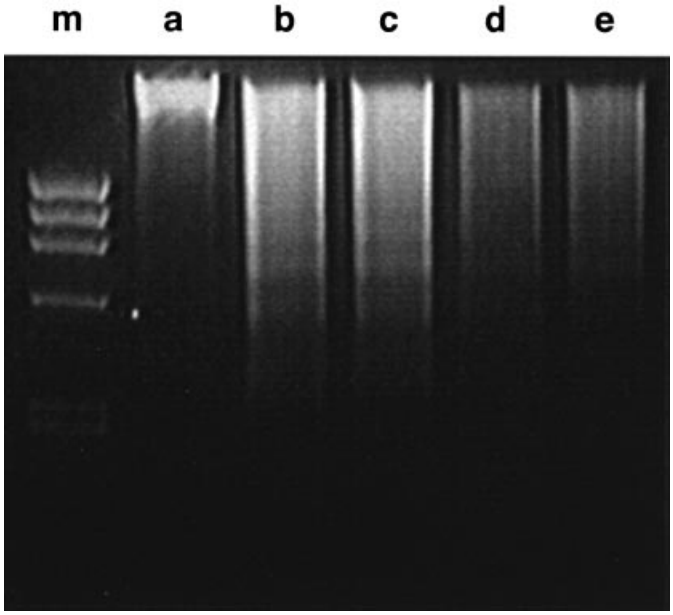

Figure 2. DNA fragmentation in HaCaT cells exposed to UVB irradiation. DNA was extracted from various cells as described under the *Materials and Methods*. Intranucleosomal DNA fragmentation was visualized by staining with ethidium bromide after agarose gel electrophoresis. *Lane a*, untreated control cells; *lane b*, 8 h after UVB exposed cells; *lane c*, 100 μM Ac-YVKD treated and 8 h after UVB exposed cells; *lane d*, 100 μM Ac-DMQD treated and 8 h after UVB exposed cells; *lane e*, 10 μM SB203580 treated and 8 h after UVB exposed cells; *lane m*, DNA molecular marker.

phosphorylation of JNK2 (55 kDa) was much less distinct compared with JNK1 (**Fig 3*C***). The significant apoptotic nuclear changes were observed at 6 h after UVB irradiation. This indicates that activation of stress-activated protein kinases (p38-MAPK and JNK) is an early event of apoptotic process in UVB-irradiated HaCaT cells.

Activation of caspase-1 and caspase-3 proteases induced by UVB irradiation Recently, caspases, especially caspase-3, are regarded as important regulators of apoptosis. To know whether the caspase activation is involved in UVB-induced apoptosis in HaCaT cells, the activities of the caspases were measured using fluorogenic substrates. The activity of caspase-3 was increased reaching a peak at 4 h after exposure to UVB in HaCaT cells (**Fig 4*A***), whereas the activation of caspase-1 was increased up to 4 h with small extent and sustained thereafter (**Fig 4*B***). The level of activation of caspase-3 was higher than that of caspase-1 in UVB-irradiated HaCaT cells.

p38-MAPK activation upstream of caspase in UVB-induced apoptosis To know the relationship between the activation of stress-activated protein kinases and caspases, the effects of the inhibitor of p38-MAPK, SB203580 were examined. The pretreatment of HaCaT cells with SB 203580 (10 μM) nearly abolished the UVB-induced phosphorylation of p38-MAPK (**Fig 5**). Under the same conditions, the effects of the inhibitor were examined for the activation of caspases. As shown in **Fig 6(*A*, *B***), the SB203580 attenuated the increase of caspase-3 activity induced by UVB irradiation. The caspase-1 activation was also suppressed by pretreatment with the inhibitor.

As caspase-3 is known to cleave a substrate protein PARP, we have examined whether UVB irradiation causes PARP cleavage in HaCaT cells. PARP was found to be cleaved from its intact 116 kDa form into the 85 kDa fragment at 4 h after exposure to UVB (**Fig 6*C***). The pretreatment of cells with the caspase-3 inhibitor abolished the cleavage of PARP. The SB203580 reduced its cleavage to almost half, which corresponds to the level of inhibition of caspase-3 activity. These results suggest that p38-MAPK may play a part in upregulation of the activation of caspases in UVB-induced apoptosis.

It has been reported that activation of p38-MAPK and JNK are dependent on caspases (Juo *et al*, 1997; Natoli *et al*, 1997; Roulston *et al*, 1998), therefore, we have examined the effect of caspase-3 on p38-MAPK/JNK activation. The UVB-induced phosphorylations of p38-MAPK and JNK were not affected by the caspase-3 inhibitor, DMQD (**Fig 7*A***, ***B***). The caspase-1 inhibitor also had no effect on these protein kinases (data not shown), suggesting that p38-K/JNK activation was caspase-independent in HaCaT cells exposed to UVB.

Involvement of ceramide and ROS in UVB-induced apoptosis The sphingomyelin pathway, initiated by hydrolysis of sphingomyelin to generate ceramide, is thought to mediate apoptosis in response to UV irradiation (Haimovitz-Friedman *et al*, 1994). Our previous study has demonstrated that a cell-permeable ceramide (C2-ceramide) induced marked apoptosis in HaCaT cells (Iwasaki-Bessho *et al*, 1998). In this context, changes in levels of ceramide during UVB-induced apoptosis were measured in HaCaT cells by the enzymatic assay with *E. coli* diacylglycerol kinase. No significant production of ceramide was observed in UVB-irradiated cells within 24 h after exposure to UVB (**Fig 8*A***). Furthermore, we have measured activities of SMases (neutral and acid) in the membrane fractions from HaCaT cells untreated or exposed to UVB. In the untreated control membranes, the activity of acid SMase was 7-fold higher than that of neutral SMase (**Fig 8*B***). No increases but rather decreases in both neutral and acid SMase activities were observed in the membrane fractions at 3 h after UVB-irradiation. These results indicated that the sphingomyelin pathway is not involved in UVB-mediated apoptosis in HaCaT cells.

It has been reported that oxidative damage occurs as a consequence of UVB irradiation of keratinocytes (Stewart *et al*, 1996). To see the possible involvement of reactive oxygens in the UVB-induced apoptosis of HaCaT cells, the effects of antioxidants were examined in the UVB-induced caspase activation. Pretreatments of the cells with various antioxidants, such as 1,10-phenanthroline, pyrrolidinedithiocarbamate and *N*-(2-mercaptopropionyl)-glycine and trolox were without any effects on the UVB-induced caspase-

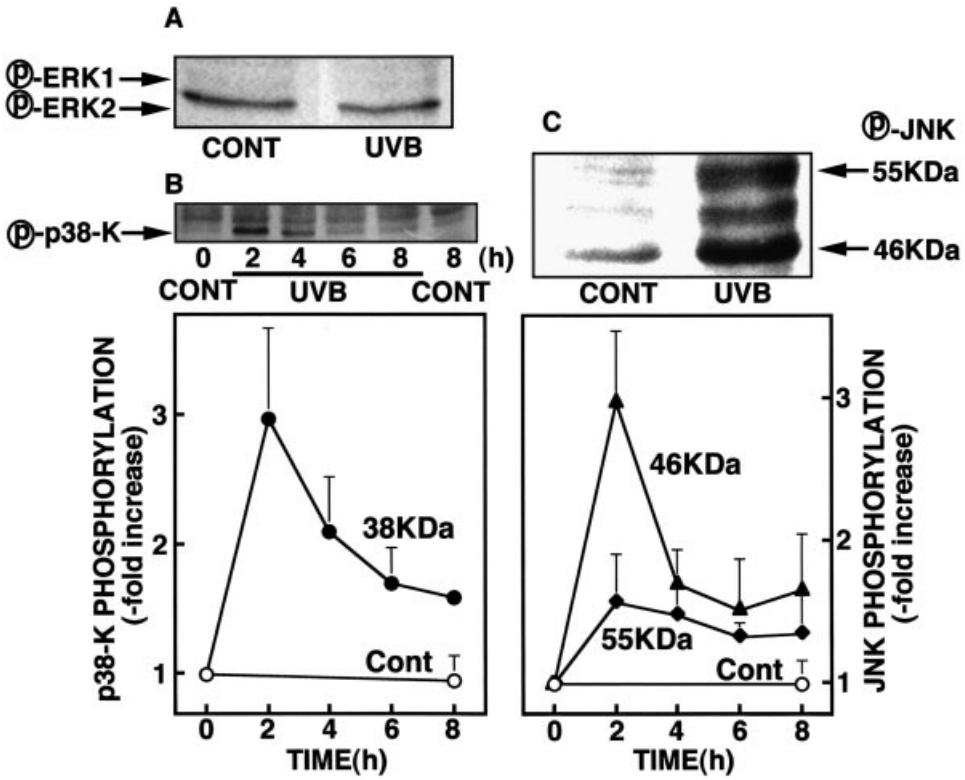

Figure 3. Phosphorylation of p38-MAPK and JNK induced by UVB irradiation. HaCaT cells were untreated (Cont) or irradiated with UVB (0.25 J per cm^2) (*closed symbol*) and were harvested at the indicated times. The cells were lysed by sonication and the lysates were subjected to SDS–polyacrylamide electrophoresis, and blotted with antiphospho-p38-MAPK (*A*) and phospho-JNK (*B*) antibodies as described under the *Materials and Methods*. The densitometric measurements of phospho-p38-MAPK (38 kDa), phospho-JNK1 (46 kDa) and phospho-JNK2 (55 kDa) were expressed as mean ± SD of at least three experiments.

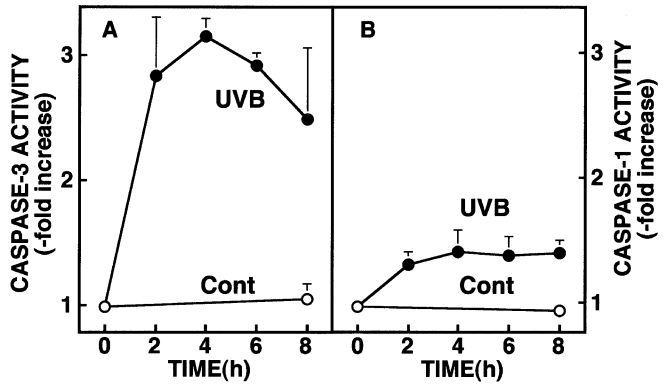

Figure 4. Activation of caspase-1 and caspase-3 induced by UVB irradiation. HaCaT cells were untreated (Cont) or irradiated with UVB (0.25 J per cm^2) (UVB) and harvested at the indicated times. The cells were lysed by sonication and the supernatants were separated by centrifugation at 100,000 × *g* for 60 min. The supernatants were incubated with caspase-1 substrate (YVAD-AFC) or caspase-3 substrate (Z-DEVD-AFC) at 30°C for 30 min. Levels of released AFC were measured using a spectrofluorometer as described under *Materials and Methods*. Data are mean ± SD from three independent experiments, each performed in triplicate.

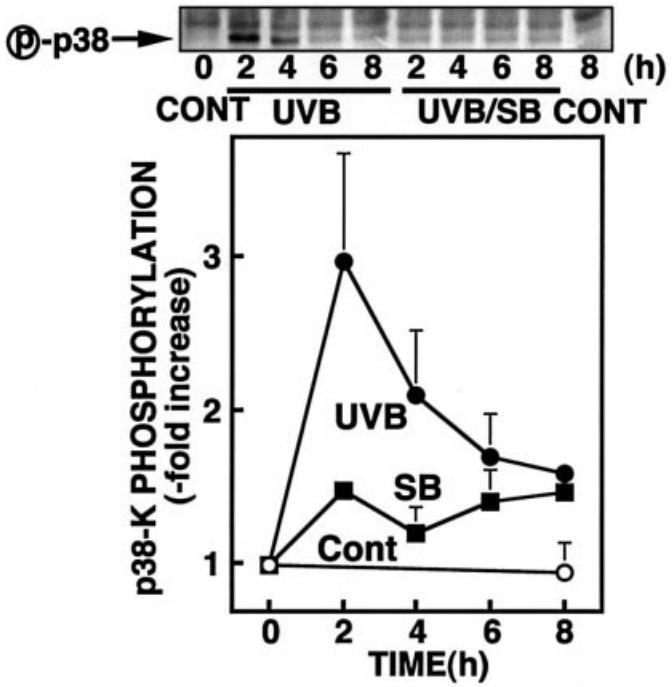

Figure 5. Inhibition of UVB-induced phosphorylation of p38-MAPK by SB203580. HaCaT cells were incubated in the absence (UVB) or presence of 10 μM SB203580 (SB) for 1 h prior to UVB irradiation (0.25 J per cm^2). The cells were harvested at the indicated times and lysed by sonication. The lysates were subjected to SDS–polyacrylamide gel electrophoresis, and analyzed by western blotting using anti-phospho-p38-MAPK antibody as described under the *Materials and Methods*. The densitometric measurements of phospho-p38-MAPK (38 kDa) were expressed as mean ± SD from three different experiments.

3 activation (data not shown). These results suggested that ROS were not implicated in UVB-induced apoptosis in HaCaT cells.

DISCUSSION

We have demonstrated that UVB exposure of human keratinocyte HaCaT cells induced apoptotic nuclear changes and activation of p38-MAPK, JNK, and caspases. The apoptotic changes of nuclei were prevented by the caspase-3 inhibitor Ac-DMQD-CHO and the p38-MAPK inhibitor SB20380, suggesting that activation of caspase-3 and p38-MAPK play important parts in inducing apoptosis in UV-irradiated HaCaT cells. Recent studies have demonstrated that UVC irradiation leads to a rapid increase in ceramide formation in U937 cells (Verheij *et al*, 1996), and that the ceramide generation is mediated by acid SMase activation in lymphoblast cells and also that exogenous ceramides induce JNK activation (Huang *et al*, 1997; Brenner *et al*, 1998). Furthermore, it was shown that treatment of Jurkat T lymphocyte cells with Fas or with synthetic ceramides caused Ras-dependent and Rac1-dependent stimulation

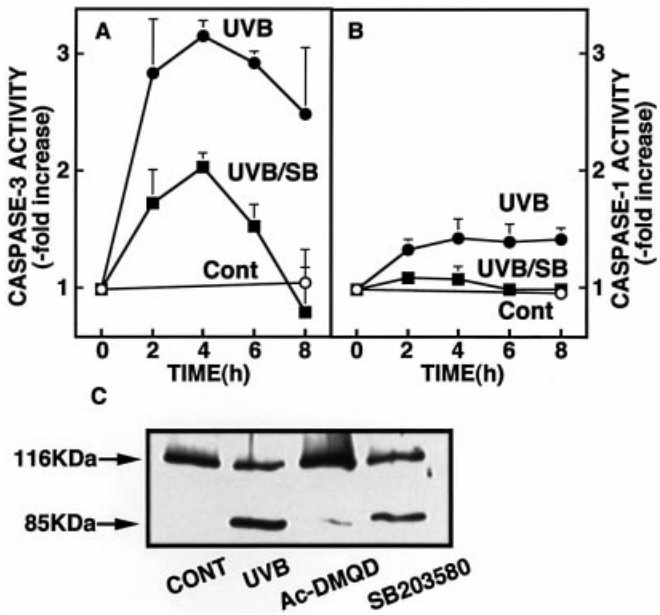

Figure 6. Effects of p38-MAPK inhibitors on UVB-induced caspase activation and PARP cleavage. HaCaT cells were treated with or without 10 μM SB203580 (*A–C*) and 100 μM Ac-DMQD (*C*) for 1 h prior to UVB irradiation (0.25 J per cm^2) and harvested at the indicated times (*A*, *B*) or at 2 h (*C*). The cells were lysed by sonication and the lysates were clarified by centrifugation and the supernatants were incubated with caspase-1 substrate (YVAD-AFC) or caspase-3 substrate (Z-DEVD-AFC) at 30°C for 30 min, levels of released AFC were measured using a spectrofluorometer and as described under *Materials and Methods*. (*C*) The lysates were subjected to SDS–polyacrylamide gel electrophoresis, and blotted with anti-PARP antibody as described under the *Materials and Methods*. Data are representative from three independent experiments. The *arrows* indicate the mobilities of full-length (116 kDa) PARP and cleavage product (85 kDa).

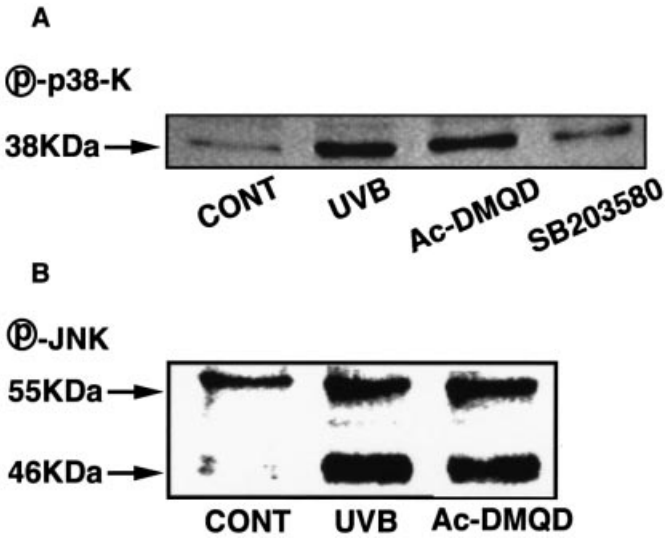

Figure 7. Effects of caspase-3 inhibitor on UVB-induced phosphorylation of p38-MAPK and JNK. HaCaT cells were treated with or without 10 μM SB203580 and 100 μM Ac-DMQD for 1 h prior to irradiation with UVB (0.25 J per cm^2) and were harvested at 4 h (*A*) or 2 h (*B*). The cells were lysed by sonication and the lysates were subjected to SDS–polyacrylamide electrophoresis, and blotted with antiphospho-p38-MAPK (*A*) and phospho-JNK (*B*) antibodies as described under the *Materials and Methods*. The data are representative from three independent experiments.

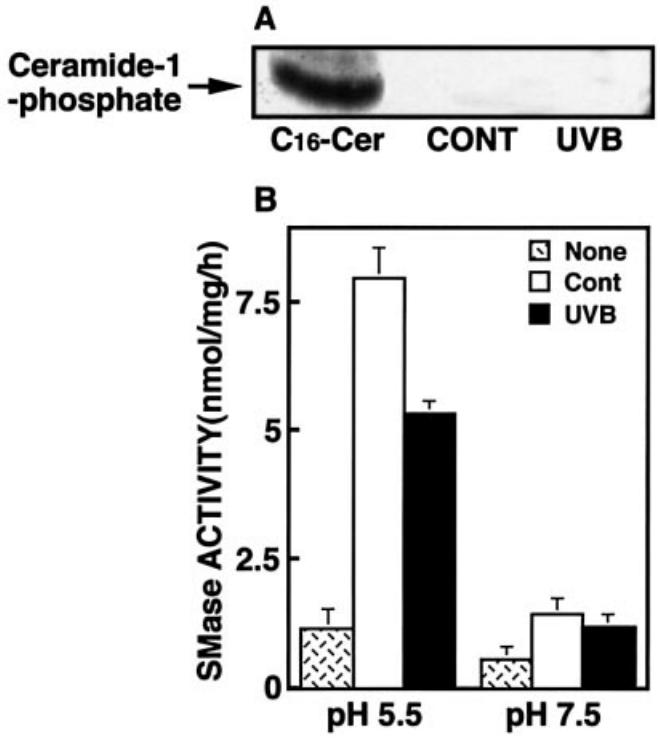

Figure 8. Changes of ceramide formation and SMase activities induced by UVB irradiation. (*A*) The levels of ceramide in HaCaT cells exposed to UVB irradiation. HaCaT cells were treated with or without UVB irradiation (0.25 J per cm^2) and harvested at 3 h. Ceramide contents were measured by the *E. coli* diacylglycerol kinase assay as described under *Materials and Methods*. (*B*) Activities of acid and neutral sphingomyelinase in HaCaT cells exposed to UVB irradiation. HaCaT cells were exposed to UVB irradiation (0.25 J per cm^2) and harvested at 3 h. The membrane fractions were prepared from UVB-exposed and untreated control cells. Acid and neutral SMase activities were measured in the membrane fractions as described under *Materials and Methods*. Data are mean ± SD from three independent experiments, each performed in duplicate.

of JNK/p38-MAPK and that the Fas-mediated or C6-ceramide-mediated apoptosis was completely inhibited by transfection of dominant inhibitory Ras and Rac1, or by a specific inhibitor of p38-MAPK, SB203580, suggesting that the JNK/p38-MAPK pathway plays an important part in the regulation of Fas-mediated apoptosis (Brenner *et al*, 1997). Our previous study has demonstrated that C2-ceramide induced marked apoptosis in HaCaT cells (Iwasaki-Bessho *et al*, 1998). As clearly shown in this study, however, neither ceramide formation nor SMase (acid and neutral) activation was observed in UVB-irradiated HaCaT cells. Also, C2-ceramide treatment did not induce activation of JNK and p38-MAPK (data not shown). These findings indicate that UV-induced JNK and p38-MAPK activation are independent of sphingomyelin hydrolysis in HaCaT cells.

Several studies have shown that at the early stage after UV exposure, production of ROS occurs in irradiated tissues (Dixit *et al*, 1983; Pelle *et al*, 1990). Various antioxidants, however, had no effects on the caspase activation induced by UVB irradiation in HaCaT cells, suggesting that ROS would not be involved in UVB-induced caspase activation.

Caspases such as caspase-1 (ICE-like) and caspase-3 (CPP32-like) that are cysteine proteinases, are known to be involved in apoptotic process induced by Fas, tumor necrosis factor-α, and anti-cancer agents in various cell systems (Enari *et al*, 1996). In HaCaT cells, autocrine release of tumor necrosis factor-α is shown to contribute at least in part to UV-induced apoptosis (Schwarz *et al*, 1995). We examined the Fas level in the UVB-irradiated HaCaT cells, but its expressed level was not changed when

examined by western blot analysis (data not shown). More recently it has been reported that UV light directly stimulates CD95 independently of CD95 ligand at the plasma membrane of the HaCaT cells (Aragane *et al*, 1998). Caspase-3 was activated by UVB exposure and caspase inhibitor, Z-VAD blocked UV-induced apoptosis. Our study has demonstrated that exposure of HaCaT cells to UVB induced significant activation of caspase-3 and much less increase of caspase-1 activity, and also that caspase-3 inhibitor, Ac-DMQD, but not caspase-1 inhibitor, Ac-YVKD, suppressed UVB-induced apoptosis, thus suggesting that caspase-3 is involved in UVB-induced apoptosis in HaCaT cells.

UV-mediated apoptosis induces activation of stress-activated protein kinases, JNK and p38-MAPK in various types of cells. Keratinocytes respond to UVB irradiation through the rapid activation of the JNK1 pathway (Ramaswamy *et al*, 1998). The relationship between caspases and the stress-activated protein kinases, however, is still controversial. It has been reported that the Fas-dependent and tumor necrosis factor-α-dependent activation of p38-MAPK and JNK require caspase activation (Juo *et al*, 1997; Natoli *et al*, 1997). Furthermore, recent study has shown that in tumor necrosis factor-α-induced apoptosis, the stress-activated protein kinases are activated in two phases; the early JNK and p38-MAPK activation is caspase-independent, but the late phase activation is caspase-dependent in murine fibroblasts (Roulston *et al*, 1998). On the other hand, in myeloid leukemia U937 cells, JNK1 positively regulates the activation of ICE-like protease during apoptosis induced by anti-cancer drugs (Seimiya *et al*, 1997). Furthermore, the other study has demonstrated that specific inhibitor SB202190 of p38α-MAPK and p38β-MAPK rather stimulated the activity of caspase-3 and potentiated apoptosis induced by Fas ligand or UV irradiation in T lymphocytes Jurkat cells (Nemoto *et al*, 1998). It was also shown that p38α-MAPK induced cell death whereas p38β-K suppressed it. In our study, pretreatment of HaCaT cells with p38-MAPK inhibitor, SB203580 reduced UVB-induced apoptosis and partially suppressed the caspase-3 and caspase-1 activation and cleavage of PARP. These results lead us to suggest that p38-MAPK may be involved in apoptosis exposed to UVB and may act upstream of caspases in HaCaT cells. Furthermore, as the caspase-3 inhibitor had no effect on JNK/p38-MAPK activation, it was assumed that UVB-induced JNK/p38-MAPK activation was independent of caspases in HaCaT cells. The discrepancy in relationship between JNK/p38-MAPK and caspases cannot be properly explained at the present time, but it may be due to differences in cell type or p38-MAPK isoform.

In normal keratinocytes, it has been reported that UVA and UVB irradiation induces ERK and JNK activation (Englaro *et al*, 1998). We did not observe ERK activation in UVB-exposed HaCaT cells. The role of ERK activation in UV responses has not yet been clearly understood. UV-induced skin injury, however, is followed by an enhanced prostaglandin synthesis that contributes to the inflammatory response in which ERK activates phospholipase A_2.

In summary, it has been shown in this study that the UVB irradiation-induced apoptosis process was independent of ceramide and ROS and further that p38-MAPK acts upstream of caspase-3.

We gratefully thank Dr. Fusenig (German Cancer Research Center) and Dr. Kuroki (University of Tokyo) for the kind gift of HaCaT cells.

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