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Sphingosine-1-Phosphate-Induced ERK Activation Protects Human Melanocytes from UVB-Induced Apoptosis

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Ultraviolet B (UVB) is known to induce apoptosis in human melanocytes. Here we show the cytoprotective effect of sphingosine-1-phosphate (S1P) against UVB-induced apoptosis. We also show that UVB-induced apoptosis of melanocytes is mediated by caspase-3 activation and poly(ADP-ribose) polymerase (PARP) cleavage, and that S1P prevents apoptosis by inhibiting this apoptotic pathway. We further investigated three major mitogen-activated protein (MAP) kinases after UVB irradiation. UVB gradually activated c-Jun N-terminal kinase (JNK) and p38 MAP kinase, while extracellular signal-regulated protein kinase (ERK) was inactivated transiently. Blocking of the p38 MAP kinase pathway using SB203580 promoted cell survival and inhibited the activation of caspase-3 and PARP cleavage. These results suggest that p38 MAP kinase activation may play an important role in the UVB-induced apoptosis of human melanocytes. To explain this cytoprotective effect, we next examined whether S1P could inhibit UVB-induced JNK and p38 MAP kinase activation. However, S1P was not found to have any influence on UVB-induced JNK or p38 MAP kinase activation. In contrast, S1P clearly stimulated the phosphorylation of ERK, and the specific inhibition of the ERK pathway using PD98059 abolished the cytoprotective effect of S1P. Based on these results, we conclude that the activation of p38 MAP kinase plays an important role in UVB-induced apoptosis, and that S1P may show its cytoprotective effect through ERK activation in human melanocytes.

Key words: Sphingolipid, Human melanocytes, ERK, UVB, Apoptosis

INTRODUCTION

Sphingosine-1-phosphate (S1P) is a multifunctional lipid mediator, which regulates cell growth, differentiation, and programmed cell death (Pyne and Pyne, 2000; Spiegel, 1999; van Koppen *et al.*, 2001). Additionally, we recently found that S1P also regulates melanogenesis via ERK activation in melanocytes (Kim *et al.*, 2003). In particular, S1P has been reported to protect cells from apoptosis in many different cell types (Cuvillier *et al.*, 1998; Edsall *et al.*, 1997; Karliner *et al.*, 2001; Kleuser *et al.*, 1998). The skin is composed of various compartments that interconnect anatomically and interact functionally. In the skin, the epidermis is mainly comprised of keratinocytes and dendritic cells, such as melanocytes and Langerhans cells, and S1P is known to promote keratinocyte survival against the apoptotic cell death induced by ceramide, ultraviolet B (UVB), or tumor necrosis factor-α (Manggau *et al.*, 2001). Moreover, it has been reported that S1P protects the pheochromocytoma cell line PC12 from apoptosis induced by serum withdrawal (Edsall *et al.*, 1997). Because both melanocytes and PC12 cells have the similar neuronal phenotype, it is possible that S1P could act as a survival factor for melanocytes. However, the effects of S1P on the survival of primary cultured human melanocytes have not been investigated.

Vitiligo is characterized by a loss of melanocytes from the epidermis, which results in the depigmentation of skin (Njoo and Westerhof, 2001). It has been suggested that apoptosis is one of the mechanisms for removing melanocytes from the skin (Huang *et al.*, 2002). UV radiation is known to induce the characteristic apoptotic cell

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death of melanocytes (Zhai *et al.*, 1996). We also observed that UV radiation induces the apoptosis of human melanocytes (Kim *et al.*, 2000) and human melanoma cells (Park *et al.*, 2000). Because S1P is a known survival factor in many cell types, we decided to examine the effect of S1P against UVB-induced apoptosis in human melanocytes.

Mitogen-activated protein (MAP) kinases are known to be highly implicated in the signal transduction of cell proliferation, differentiation, stress response, and apoptosis (Bevan, 2001; Marshall, 1995). The family of MAP kinases includes extracellular signal-regulated protein kinase (ERK), p38 MAP kinase, and c-Jun N-terminal kinase (JNK) (Chang and Karin, 2001). The ERK signal transduction pathway is a major signaling cascade that plays a major role in cell proliferation and differentiation and in cell survival as mediated by various growth factors (Kolch, 2000; Marshall, 1995). In contrast, the p38 MAP kinase and the JNK pathways can be stimulated by various inflammatory cytokines, free radicals, UV, heat shock, osmotic shock, ischemia, and many other factors (Harper and LoGrasso, 2001; Zanke *et al.*, 1996). Thus, apoptosis is generally thought to be mediated through the activation of p38 MAP kinase and JNK with little change or reduction in the activity of ERK.

In this study, we investigated the UV-induced apoptosis of melanocytes and found that S1P can protect human melanocytes against apoptotic cell death. In order to understand the signaling mechanism of melanocyte apoptosis, changes of signaling kinases were investigated after UVB irradiation and S1P treatment. UV irradiation has been shown to activate various MAP kinases (Butterfield *et al.*, 1997; Peus *et al.*, 1999). However, the activations of each MAP kinase have been reported to be UV wavelength and cell type dependent (Peus *et al.*, 1999; Tada *et al.*, 2002; Yanase *et al.*, 2001; Zanke *et al.*, 1996). Our results, obtained using human melanocytes show that UVB irradiation leads to the sustained activation of JNK and p38 MAP kinase, while ERK is transiently inactivated. Moreover, whereas S1P did not suppress the activation of JNK or p38 MAP kinase, it induced the activation of ERK. Further experiments using the specific ERK pathway inhibitor, PD98059, indicated that S1P-induced ERK activation may be responsible for melanocyte survival.

MATERIALS AND METHODS

Materials

S1P was purchased from Alexis (San Diego, CA); LY294002, SB203580, and SP600125 from Calbiochem (San Diego, CA); PD98059 from Cell Signaling Technology (Beverly, MA); fatty acid-free bovine serum albumin (BSA), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and cholera toxin (CT) from Sigma (St. Louis, MO). S1P was added to cells as a complex with 0.4% BSA. Antibodies recognizing phospho-specific ERK1/2 (Thr202/Tyr204, number 9101S), phospho-specific p38 MAP kinase (Thr180/Tyr182, number 9211S), and total (phosphorylated and non-phosphorylated) ERK1/2 (number 9102) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). PARP antibody was purchased from BD Pharmingen (San Diego, CA) and phospho-specific JNK1/2 (Thr183/Tyr185, G-7, sc-6254), total JNK2 (D-2, sc-7345), total p38 MAP kinase (A-12, sc-7972), caspase-3 (sc-7272), and actin (I-19) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell cultures

Human epidermal melanocytes were isolated from adolescent foreskins, as previously described (Eisinger and Marko, 1982). The cells were maintained in modified MCDB 153 (Sigma, St. Louis, MO) as previously described (Medrano and Nordlund, 1990), supplemented with 5% FBS (Hyclone, Logan, UT), 13 μg/mL bovine pituitary extract (Gibco BRL, Gaithersburg, MD), 10 ng/mL 12-*O*-tetradecanoylphorbol-13-acetate (Sigma), 5 μg/mL insulin (Sigma), 0.5 μg/mL transferrin (Sigma), 1 μg/mL tocopherol (Sigma), 0.5 μg/mL hydrocortisone (Sigma), 1 ng/mL human recombinant basic fibroblast growth factor (Gibco BRL), and 1% penicillin-streptomycin (10,000 U/mL and 10,000 μg/mL, respectively) (Gibco BRL). Cells were maintained in a humidified incubator in 5% CO_2 at 37°C. Second and third passage melanocytes were used in the experiments.

Cell viability assay and microscopy

Cell viability was determined using a crystal violet assay (Dooley *et al.*, 1994). After incubating with the test substances for 24 h, the culture medium was removed. Cells were then stained with 0.1% crystal violet in 10% ethanol for 5 min and rinsed four times. The crystal violet retained by adherent cells was then extracted with 95% ethanol. Absorbance was determined at 590 nm using an ELISA reader (Molecular Devices, Sunnyvale, CA). Before cell viability was measured, the cells were observed under a phase contrast microscope (Olympus Optical Co., Tokyo, Japan) and photographed with a digital color video camera TK-C1380 (JVC, Yokohama, Japan) supported by Image-Pro® Plus software (Media Cybernetics, Silver Spring, MD).

UVB irradiation

Human melanocytes were plated in 6-well plates. The medium was replaced by 1 mL of phosphate-buffered saline (PBS), and the cells were irradiated once with a UVB source (BLE-1T158, Spectronics corp., Westbury, NY). A Kodacel filter (TA401/407. Kodak, Rochester, NY)

was used to remove wavelengths of less than 290 nm (UVC). The applied energy was measured with a Waldmann UV meter (model No. 585100; Waldmann Co., VS-Schwenningen, Germany). Immediately after irradiation, the transferred medium was returned to each well. The irradiated cells were maintained at 37°C in a 5% CO_2 atmosphere.

Western blot analysis

Cells were grown in 100 mm culture dishes, starved of serum for 24 h, and treated with test substances as indicated. They were then lysed in cell lysis buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, protease inhibitors (Complete™, Roche, Mannheim, Germany), 1 mM Na_3VO_4, 50 mM NaF, and 10 mM EDTA]. Ten micrograms of protein per lane was separated by SDS-polyacrylamide gel electrophoresis and blotted onto PVDF membranes, which were saturated with 5% dried milk in Tris-buffered saline containing 0.4% Tween 20. Blots were incubated with the appropriate primary antibodies at a dilution of 1:1000, and then further incubated with horseradish peroxidase-conjugated secondary antibody. Bound antibodies were detected using an enhanced chemiluminescence plus kit (Amersham International, Little Chalfont, U.K.).

Statistics

Differences between results were assessed for significance using the Students *t*-test.

RESULTS

Cytoprotective effect of S1P against UVB-induced apoptosis in human melanocytes

Previously, we reported that UVB could induce apoptosis in human melanocytes (Kim *et al.*, 2000) and in G361 human melanoma cells (Park *et al.*, 2001). To determine the cytoprotective effect of S1P on human melanocytes, cells were exposed to increasing doses of UVB with or without SIP pretreatment. Twenty-four hours after UVB irradiation, cells were photographed under a phase contrast microscope (Fig. 1A). We observed that a significant portion of the unpretreated cells died after UVB irradiation, and that S1P pretreated cells showed robust dendrites

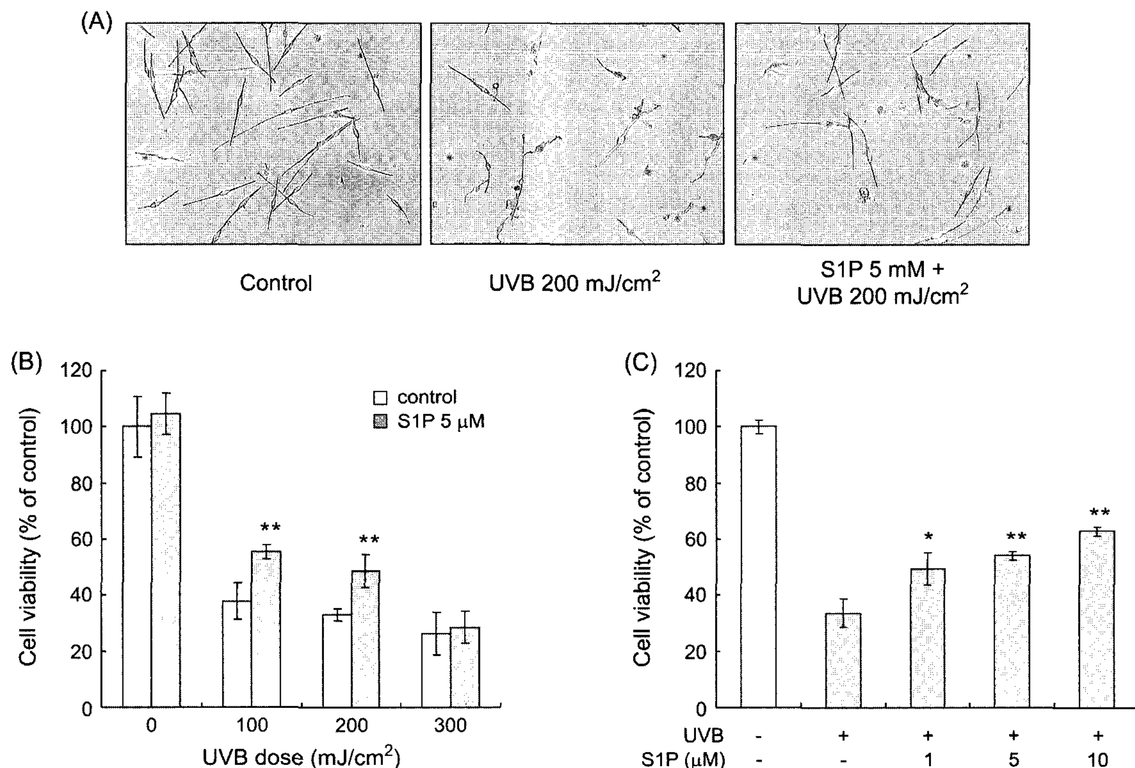

Fig. 1. Cytoprotective effect of S1P against UVB-induced apoptosis in human melanocytes. After serum starvation, the cells were pretreated with 5 μM of S1P for 1 h. Then UVB was irradiated at the indicated doses. (A) After 24 h, phase contrast pictures were taken using a color video camera and (B) the viability of the cells was measured by crystal violet assay. (C) The cells were pretreated with the indicated concentrations of S1P for 1 h. Twenty-four hours after UVB irradiation (200 mJ/cm²), a crystal violet assay was performed. Data represent the means±SD of triplicate assays expressed as a percent of control. The experiment was repeated three times independently, and produced similar results. **$P<0.01$, *$P<0.05$ compared to UVB control.

and more cells were attached to the culture dish, indicating that more cells survived. When cell viability was measured by crystal violet assay, it was found that UVB irradiation induced significant cell death as shown in Fig. 1B. In agreement with the morphological observation, a significant cytoprotective effect was associated with 5 μM S1P pretreatment (Fig. 1B). However, S1P was not cytoprotective against a high dose of UVB (300 mJ/cm^2), but a similar cytoprotective effect was observed against a low dose of UVB (<100 mJ/cm^2) (data not shown). We next tested the effect of S1P at concentrations ranging from 1-10 μM, and observed a dose-dependent cytoprotective effect (Fig. 1C).

Effects of S1P on caspase activation and PARP cleavage induced by UVB

We next examined whether S1P has inhibitory effects on caspase activation and PARP cleavage, which are deeply involved in apoptosis. Our results showed that UVB irradiation activated caspase-3, so that the precursor forms disappeared in a UVB-dependent manner (Fig. 2). Furthermore, UVB irradiation also led to proteolytic cleavage of PARP. Thus, we found that the 116-kDa full-length PARP was converted to the apoptotic 85-kDa fragment. Therefore, it was of interest to us to determine whether S1P could inhibit caspase activation and PARP cleavage, since S1P partially protected the cytotoxic effect of UVB. As was found in experiments using Jurkat T-lymphocytes (Cuvillier *et al.*, 1998), S1P pretreatment markedly reduced the cleavage of PARP and the activation of caspase-3 (Fig. 2).

Effects of UVB Irradiation on the MAP Kinases

To investigate the mechanism of UVB-induced melanocyte apoptosis, we examined the regulatory effect of UVB on three major MAP kinases. We were first interested in the activations of JNK and p38 MAP kinase to determine the effects of UVB on the stress regulated kinases. As shown in Fig. 3, UVB irradiation (200 mJ/cm^2) resulted in the activations of JNK and p38 MAP kinase after 10 min. The JNK and p38 MAP kinase activated markedly at 30 min and persisted at least up to 180 min. We also examined the ERK signaling pathway (Fig. 3).

Effect of SP600125 and SB203580 on UVB-induced apoptosis

To test the possible roles of JNK and p38 MAP kinase in UVB-mediated apoptosis, cells were pretreated with SP600125 (20 μM) or SB203580 (5 μM), specific JNK or p38 MAP kinase inhibitors, respectively. As shown in Fig. 4A, SB203580 promoted the survival of melanocytes, but SP600125 was not effective against UVB-induced cell death. We further investigated whether these specific inhibitors really blocked the JNK or p38 MAP kinase pathways. In the event, SB203580 (5 μM) inhibited p38 MAP kinase activation, while SP600125 (20 μM) failed to block the JNK pathway in melanocytes (Fig. 4B). We next examined the effects of SP600125 and SB203580 on the caspase activation and PARP cleavage induced by UVB. As expected, SB203580 inhibited caspase-3 activation and PARP cleavage (Fig. 4C). In contrast, SP600125 rather facilitated the apoptotic signaling pathways. These results indicate that p38 MAP kinase is important in the UVB-mediated apoptosis of melanocytes. However, the role of JNK needs further investigation.

Effects of S1P on the MAP Kinases

To explain its cytoprotective effect, we investigated whether S1P could affect UVB-induced stress-activated kinases. Thus, we further investigated the effect of S1P

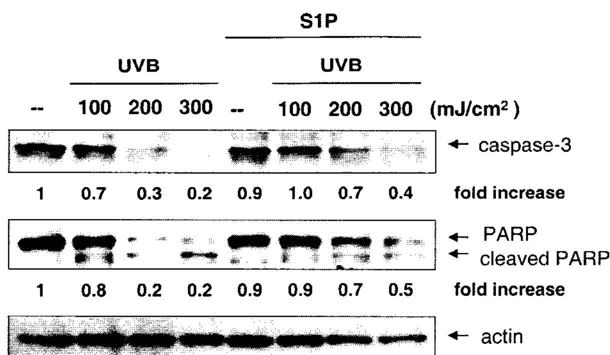

Fig. 2. Effects of S1P on the UVB-induced activation of caspases and the cleavage of PARP. Cells were irradiated with varying doses of UVB in the absence or presence of S1P. Whole cell lysates were then subjected to Western blot analysis with antibodies against caspase-3 and PARP. Equal protein loading was verified using actin antibody.

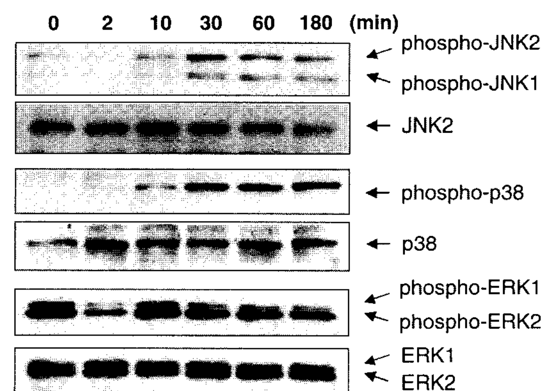

Fig. 3. Effects of UVB on the MAP kinase pathways. (A) After serum starvation, the cells were irradiated with 200 mJ/cm^2 of UVB. Samples were collected at the time points indicated after UVB irradiation. Cell lysates were then subjected to Western blot analysis with antibodies against phospho-specific MAP kinases. Equal protein loadings were checked by reaction with phosphorylation-independent MAP kinase antibodies.

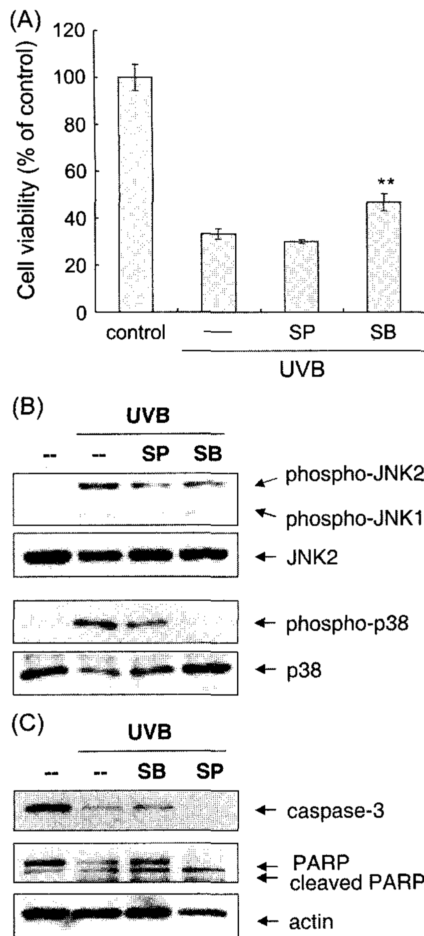

Fig. 4. SB203580 (SB) protects cells from UVB irradiation. After serum starvation, the cells were pretreated with 5 μM of S1P in the absence or presence of SB203580 (5 μM) or SP600125 (20 μM) for 1 h. Then 200 mJ/cm² of UVB was irradiated (A) After 24 h, the viability of the cells was measured by crystal violet assay. Data represent the means±SD of triplicate assays expressed as a percent of control. **P< 0.01 compared to the UVB control. (B) One hour after 200 mJ/cm² of UVB irradiation, cells were harvested. Cell lysates were then subjected to Western blot analysis with antibodies against phospho-specific JNK and p38 MAP kinase. Equal protein loadings were confirmed by reaction with phosphorylation-independent JNK and p38 MAP kinase antibodies (C) Twenty-four hours after applying 200 mJ/cm² of UVB irradiation, cells were collected. Whole cell lysates were then subjected to Western blot analysis with antibodies against caspase-3 and PARP. Equal protein loading was verified using actin antibody.

on the activation of ERK, which are known to be involved in the cell proliferation and survival signaling cascade. Indeed, S1P was found to stimulate the phosphorylation of ERK as shown by a time course experiment using a phospho-specific antibody against ERK (Fig. 5A). Phosphorylation of ERK was observed immediately after incubation with 10 μM S1P, reached a maximum 30 min after incubation, and decreased to the basal level within 180 min (Fig. 5A). To further investigate the involvement

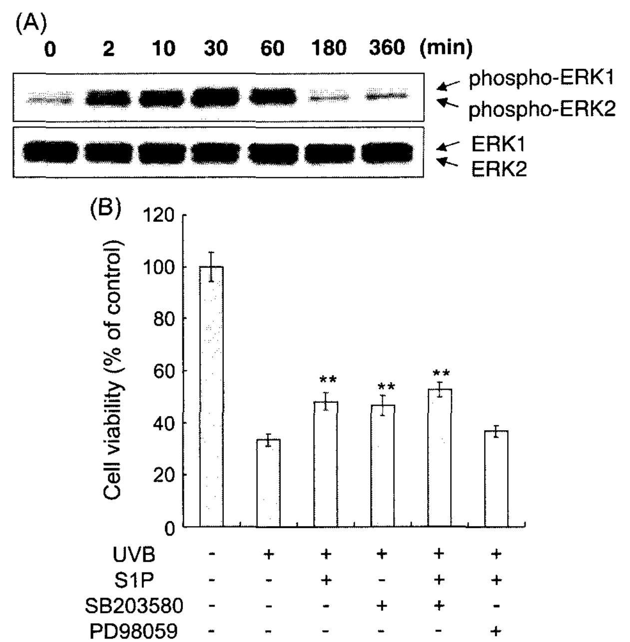

Fig. 5. Effects of S1P after UVB irradiation. (A) The cells were stimulated with 10 μM S1P at the times indicated. Cell lysates were then subjected to Western blot analysis with antibodies against phospho-specific ERK. Equal protein loading was confirmed by reaction with phosphorylation-independent ERK antibody. Experiments were performed three times and representative results are shown. (B) After serum starvation, the cells were pretreated with 5 μM of S1P in the absence or presence of PD98059 (20 μM) or SB203580 (5 μM) for 1 h, and then irradiated with 200 mJ/cm² of UVB. After 24 h, the viability of the cells was measured by crystal violet assay. Data represent the means±SD of triplicate assays expressed as a percent of the untreated control. **P<0.01 compared to UVB-treated control.

of the ERK signaling cascade in S1P-mediated cell survival, we used PD98059, a specific ERK pathway inhibitor. Interestingly, PD98059 pretreatment (20 μM) abrogated the anti-apoptotic effect of S1P (Fig. 5B). These results suggest that the ERK pathway may play an important role in S1P-mediated cell survival. Based on these results, we conclude that S1P may show its cytoprotective effect through ERK activation, although S1P has no influence on JNK or p38 MAP kinase activation. As shown in Fig. 5B, S1P showed only partial anti-apoptotic effect.

DISCUSSION

It has been reported that S1P suppresses the apoptosis of many cell types, including Jurkat T cells (Cuvillier *et al.*, 1998), HL-60 cells (Kleuser *et al.*, 1998), U937 cells (Cuvillier *et al.*, 1996), human keratinocytes (Manggau *et al.*, 2001), human umbilical vein endothelial cells (Kwon *et al.*, 2001), and rat pheochromocytoma PC12 cells (Edsall *et al.*, 1997). The epidermal melanocytes of human skin are derived from the neural crest and posses the metabolic ma-

chinery required for the synthesis of melanin. Melanocytes and PC12 cells share the similar neuronal phenotype. Nerve growth factor (NGF) is known to protect PC12 cells against serum withdrawal, cisplatin, or ceramide (Edsall *et al.*, 2001; Fischer *et al.*, 2001), and to promote the survival of melanocytes from UV-induced apoptosis (Zhai *et al.*, 1996). Interestingly, NGF increases the activity of sphingosine kinase, which catalyses the formation of S1P (Edsall *et al.*, 1997). Thus, S1P is also thought to play a role as a survival factor for melanocytes. In this study, S1P showed a significant anti-apoptotic effect in human melanocytes when cells were irradiated with UVB. It had also been reported that apoptotic cell death is mediated through the serial activation of the caspase family proteases and the clevage of the DNA repair enzyme PARP (Hengartner, 2000). We also found that S1P inhibits the activation of caspase-3 and the clevage of PARP that are hallmarks of apoptosis.

To study the molecular process of UVB-induced apoptosis and S1P-mediated cell survival in human melanocytes, we investigated the activation of MAP kinases. It has been reported that various cytotoxic agents, including UV, often activate JNK and p38 MAP kinase in a coordinated fashion (Xia *et al.*, 1995). In human melanocytes, it has been reported that UVB irradiation results in the activation of JNK and p38 MAP kinase, but not ERK (Tada *et al.*, 2002). Our results demonstrate that UVB irradiation resulted in the activation of JNK and p38 MAP kinase and a transient inactivation of ERK, whereas S1P potently stimulated the phosphorylation of ERK. JNK and p38 MAP kinase are important mediators of stress signals that ultimately lead to apoptosis (Harper and LoGrasso, 2001; Peus *et al.*, 1999; Zanke *et al.*, 1996). Additionally, NGF withdrawal is known to induce apoptosis via the sustained activation of JNK and p38 MAP kinase and via the inhibition of ERK in PC12 cells (Xia *et al.*, 1995).

No well-characterized specific inhibitor of JNK exists, though SP600125, a recently reported inhibitor of JNK, has been used to investigate the role of JNK in apoptosis of melanocytes (Bennett *et al.*, 2001; Han *et al.*, 2001). Unfortunately, SP600125 did not suppress JNK activation in melanocytes and unexpectedly potentiated the cytotoxic effect of UVB by activating caspases and cleaving PARP. In accordance with our results, it was also reported that SP600125 activates caspases and has no protective effect on anisomycin-induced cell death (Watanabe *et al.*, 2002). In addition, it was reported that SP600125 enhances apoptosis in the p53+/+ cells of a human colorectal carcinoma cell line, HCT116 (Zhang *et al.*, 2002). Very recently, SP600115 is clearly documented to inhibit several kinases non-specifically rather than selectively inhibiting JNK (Bain *et al.*, 2003). These results suggest that the role of SP600125 as a specific inhibitor of JNK is questionable. Although our results did not provide any direct evidence that JNK is related to the UVB-induced apoptosis of melanocytes, the role of JNK could not be excluded, because it was reported that the transfection of JNK enhanced cell death due to UVB irradiation (Waetzig and Herdegen, 2002). In addition, it has been proposed that S1P promotes cell survival via the inhibition of JNK activation in U937 cells (Cuvillier *et al.*, 1996). However, in our study, S1P did not suppress UVB-induced JNK activation.

We examined whether S1P inhibits the UVB-induced activation of p38 MAP kinase, but it had no effect. Thus, we used a specific p38 MAP kinase inhibitor, SB203580 to examine the role of the p38 MAP kinase pathway in UVB-induced apoptosis. We observed that inhibition of p38 MAP kinase by SB203580 resulted in the protection of cells against UVB irradiation. These results suggest that the p38 MAP kinase pathway is involved in UVB-induced apoptotic cell death. Our data indicate that the protective effects of S1P are not directly associated with the JNK and p38 MAP kinase activations induced by the UV irradiation of melanocytes.

It has been suggested that the activation of ERK, by various growth factors, promotes cell survival (Cuvillier *et al.*, 1996; Xia *et al.*, 1995). S1P has been reported to stimulate ERK in several types of cells, for example, U937 cells (Cuvillier *et al.*, 1996), HEK293 cells (Van Brocklyn *et al.*, 2000), breast adenocarcinoma MCF-7 cells (Nava *et al.*, 2002), and astrocytes (Pebay *et al.*, 2001). In our present study, S1P induced a sustained ERK activation in melanocytes, lasting at least for 1 h. Moreover, blocking the ERK pathway with PD98059 not only suppressed the S1P-induced activation of ERK, but also abrogated the cytoprotective effect of S1P against UVB-induced apoptosis, suggesting that ERK activation is responsible for cell survival by S1P.

Because S1P or SB203580 only partially protected the cells against UVB irradiation, we next tested their combined effect in order to examine the synergistic effects of ERK activation and p38 MAP kinase inhibition. Our results showed that the combination of SB203580 and S1P increased cell survival slightly. Thus, JNK involvement could explain the partial cytoprotective effect of S1P and SB203580. Finally, it should be noted that UVB induced the immediate inactivation of ERK in the melanocytes. In PC12 cells, the withdrawal of NGF induced apoptosis via sustained the activation of JNK and p38 MAP kinase and the inhibition of ERK (Xia *et al.*, 1995). We also showed that ERK activation is responsible for the cytoprotective effect of S1P in melanocytes. These results suggest that the balance between the activation of p38 MAP kinase by cytotoxic stimuli and the activation ERK by survival factors may determine the fate of the cell, i.e., whether the cell survives or undergoes apoptosis.

Taken together, our data show that ERK is involved in regulating cell survival, and that p38 MAP kinase activation is important for UVB-induced apoptosis. In conclusion, we have demonstrated that S1P protects human melanocytes from UVB-induced apoptosis and that it exerts its cytoprotective effects mainly via ERK activation.

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