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Regulation of UVB-Induced IL-8 and MCP-1 Production in Skin Keratinocytes by Increasing Vitamin C Uptake via the Redistribution of SVCT-1 from the Cytosol to the Membrane

Jae Seung Kang[1,4], Ha Na Kim[1,4], Da Jung Jung[1], Jee Eun Kim[1], Ga Hee Mun[1], Yeong Seok Kim[2], Daeho Cho[3], Dong Hoon Shin[1], Young-Il Hwang[1] and Wang Jae Lee[1]

It is well known that UVB (290–320 nm) induces inflammation in skin by the transcription and release of cytokines and chemokines from skin keratinocytes. In addition, it is considered that intracellular reactive oxygen species (ROS) plays an important role in UVB-induced inflammatory response in the skin. Therefore, we investigated the effect of vitamin C, a potent antioxidant, on the regulation of UVB-induced skin inflammation via the modulation of chemokines production. Vitamin C uptake into keratinocytes is increased by UVB irradiation in a time- and dose-dependent manner through the translocation of sodium-dependent vitamin C transporter-1 (SVCT-1), a vitamin C-specific transporter, from the cytosol to the membrane. To evaluate the effect of vitamin C on the chemokine mRNA expression, we performed RNase protection assay. As a result, there was a remarkable change in chemokine mRNA expression, especially IL-8 and monocyte chemoattractant protein (MCP)-1 expression. In addition, increased IL-8 and MCP-1 mRNA expressions were suppressed by vitamin C treatment. We also confirmed the results of protein levels measured by ELISA. Taken together, vitamin C uptake is increased in UVB-irradiated keratinocytes through the translocation of SVCT-1 and regulates inflammatory response in the skin via the downregulation of IL-8 and MCP-1 production.

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INTRODUCTION

UV ray lies in the range of wavelengths produced by the sun and causes a significant impairment of immunological function in the human skin. It is divided into three wavelength bands: UVA (320–400 nm), UVB (290–320 nm), and UVC (200–290 nm). Only 6% of solar radiation is within the UVB band, and most of this solar radiation is absorbed by ozone. Nevertheless, the UVB region is of particular interest, because UVB can not only induce damages at the molecular levels such as in DNA molecules but also lead the failure to induce cutaneous delayed-type hypersensitivity reaction via the impairment of the function of the epidermal antigen-presenting cells (Weichenthal *et al.*, 2000). UVB radiation also has diverse effects on the production of cytokines, including IL-1, IL-6, IL-7, IL-12, IL-15, and GM-CSF, from keratinocytes (de Vos *et al.*, 1993; Ariizumi *et al.*, 1995; Aragane *et al.*, 1996; Blauvelt *et al.*, 1996; Suh *et al.*, 2002).

Reactive oxygen species (ROS), including hydrogen peroxide, hydroxyl radical, superoxide anion, and nitric oxide, play a pivotal role in the intracellular signal transduction pathway, although they are highly toxic to the human body, especially to the immune system (Bogdan *et al.*, 2000). ROS can regulate not only the expression of inflammatory cytokines, such as tumor necrosis factor-α through NF-κB activation and tyrosine kinase-dependent pathway, but also cell death as a mediator of apoptosis (Schreck *et al.*, 1991; Gossart *et al.*, 1996; McGowan *et al.*, 1996). It has been reported that UVB irradiation increases intracellular ROS levels through the suppression or impairment of the activity of ROS-scavenging enzymes such as superoxide dismutase in the skin. This process results in the modulation of the immune system in the skin such as the production of inflammatory cytokines (Sasaki *et al.*, 2000).

Vitamin C is one of the most frequently investigated antioxidants, which protects the cells from the ROS-mediated damage by UVB irradiation (Stewart *et al.*, 1996; Nakamura

[1]*Department of Anatomy and Tumor Immunity Medical Research Center, Seoul National University College of Medicine, Seoul, Korea;* [2]*Department of Anatomy, Inje University College of Medicine, Seoul, Korea and* [3]*Department of Life Science and Research Center for Women's Disease, Sookmyung Women's University, Seoul, Korea*

[4]*These authors contributed equally to this work*

Correspondence: Dr Wang Jae Lee, Department of Anatomy, Seoul National University College of Medicine, 28 Yongon-dong Chongno-gu, Seoul 110-799, Korea. E-mail: kinglee@snu.ac.kr

Abbreviations: MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SVCT, sodium dependent vitamin C transporter

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et al., 1997; Reddy and Bhat, 1999). In fact, there is a report on the suppressive effect of vitamin C on proinflammatory cytokines, such as IL-1α and IL-6, from UVA-irradiated keratinocytes (Tebbe *et al.*, 1997). The vitamin C transport system through the plasma membranes is classified into two types: passive transport and active transport (Sabry *et al.*, 1958). Simple diffusion and facilitated diffusion occur by the difference in concentration between the internal and the external compartment and they belong to passive transport (Wilson and Dixon, 1989). Active transport occurs when the cells require a large amount of vitamin C in a short period of time. In addition, it is mediated by sodium-dependent vitamin C transporter (SVCT)-1/-2, a vitamin C-specific transporter (Matthias, 2002). SVCT-1 is a high-capacity, low-affinity ascorbic acid carrier. On the other hand, SVCT-2 is a low-capacity, high-affinity transporter (Daruwala *et al.*, 1999; Rajan *et al.*, 1999; Wang *et al.*, 2000). It has been reported that mild oxidative stress stimulates the transfer of vitamin C from the extracellular to the intracellular compartment. There have been few studies on the role of SVCTs in the inflammatory response induced by UVB.

It is generally accepted that chemokines are important mediators for UVB-induced inflammatory response. IL-8 is one of the most comprehensively studied chemokines and it has been characterized as a neutrophil-activating protein-1. IL-8 is upregulated in human keratinocytes following UVB-irradiation both *in vitro* (Kondo *et al.*, 1993; Stein *et al.*, 1997; Pernet *et al.*, 1999) and *in vivo* (Strickland *et al.*, 1997). Therefore, IL-8 effectively stimulates neutrophils to migrate to the inflammatory site and plays an important role in the induction of skin inflammation by UVB (Strickland *et al.*, 1997). Monocyte chemotactic protein (MCP)-1 is known as a member of a distinct subfamily of the IL-8 supergene family. It is a member of the proinflammatory CC chemokine superfamily and plays a critical role in the recruitment of monocytes and lymphocytes during inflammatory response (Baggiolini *et al.*, 1997). In contrast to IL-8, there have been few studies on the association between MCP-1 and inflammation induced by UVB irradiation. In the production of chemokines and the induction of inflammatory response, ROS is known as an important mediator. It is suggested that the effective regulation of chemokine-mediated inflammatory response can be achieved by scavenging ROS with appropriate antioxidants.

It has been known that the UVB-induced skin inflammation and skin damage are caused by the activation of p38 mitogen-activated protein kinase (MAPK) and ROS play an important role in this process (Chiu *et al.*, 2001; Zhang *et al.*, 2001; Cho *et al.*, 2005). In particular, p38MAPK activation has a critical role in IL-8 production in skin keratinocyte by UVB irradiation (Kim *et al.*, 2005). It is also involved in IL-8 and MCP-1 production in monocyte-lineage cells, eosinophils and bronchial epithelial cells (Hall *et al.*, 2005; Wong *et al.*, 2005; Zeng *et al.*, 2005). According to the report by Bowie and O'Neill (2000), p38MAPK is the intracellular target molecule of vitamin C. In addition, we have already reported that vitamin C regulates the IL-18 production via the regulation of p38MAPK activation (Cho *et al.*, 2003).

Therefore, we hypothesized that vitamin C uptake may be increased for the regulation of inflammatory response by scavenging UVB-induced ROS and p38MAPK acts as a signal transducer in this process. To test the hypothesis, we investigated the changes in sodium-dependent SVCT-1/-2 expression by increased vitamin C uptake.

RESULTS

Increase of vitamin C uptake in UVB-irradiated HaCaT

To examine the effect of UVB irradiation on vitamin C uptake into human keratinocyte cell line, HaCaT, we determined the optimal dose of UVB without cell damage. After HaCaT cells were exposed to various doses of UVB, cells were cultured for 24 hours and subjected to Annexin V/PI staining. As shown in Figure 1, more than 90% of cells were viable in doses of 50 and 100 J/m^2 of UVB, but we observed damaged cells by UVB irradiation in a dose of 150 J/m^2 of UVB. Moreover, we could observe the increase of vitamin C uptake in a dose of 100 J/m^2 (Figure 2a). We also examined the optimal incubation time, which was sufficient for HaCaT cells to uptake vitamin C, after irradiation of 100 J/m^2 of UVB. Interestingly, vitamin C uptake was saturated at 2 hours after UVB irradiation (Figure 2b).

Changes in SVCT expression by UVB irradiation

As shown in Figure 2a and b, vitamin C uptake was increased by UVB irradiation. Therefore, we determined whether the enhancement of vitamin C uptake into UVB-irradiated HaCaT cells is caused by increased expression of SVCT. To analyze SVCT mRNA transcript expression after UVB irradiation, we performed reverse transcriptase (RT)-PCR.

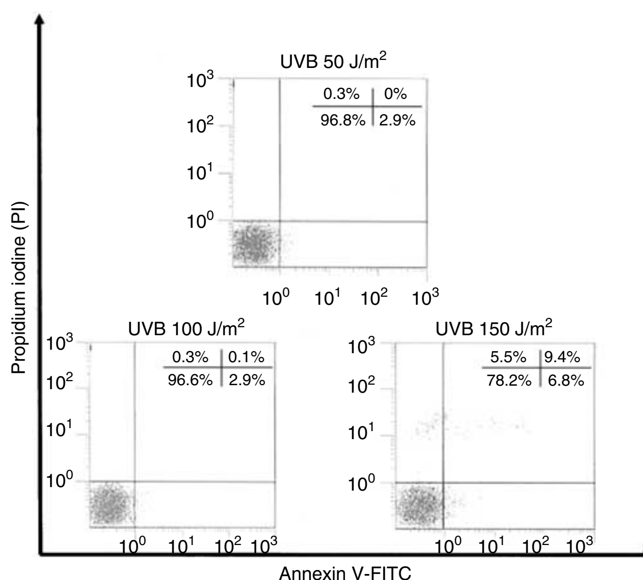

Figure 1. Determination of optimal UVB dose without damage to human keratinocyte cell line, HaCaT. HaCaT (8×10^6) cells were exposed to 50, 100, and 150 J/m^2 of UVB and then incubated for 24 hours. The cells were collected and stained with Annexin V-FITC and propidium iodide (PI). The effect of UVB on the cell viability was measured by flow cytometry. Results are representative of three experiments.

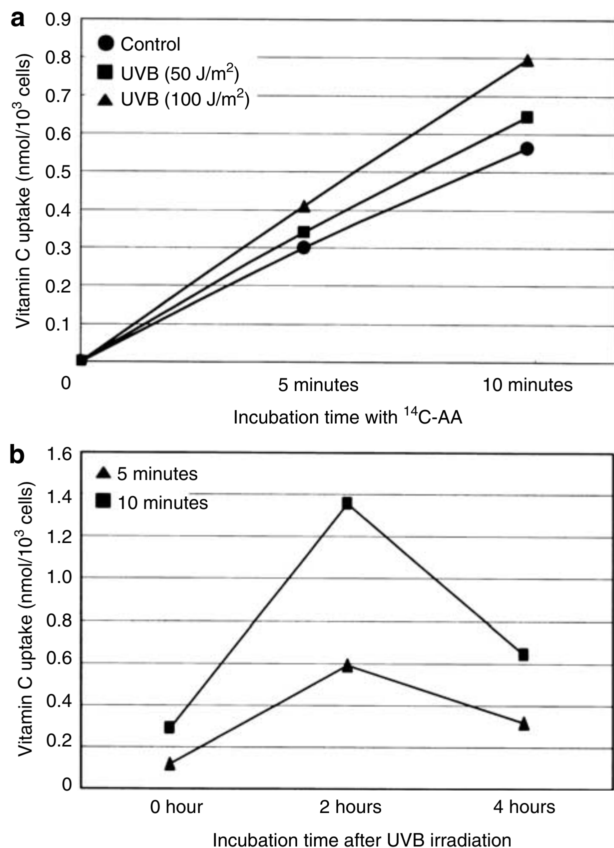

Figure 2. Effect of UVB on vitamin C uptake into human keratinocyte cell line, HaCaT. (**a**) After HaCaT (8×10^6) cells were exposed to sublethal doses of UVB (50 and 100 J/m^2), cells were incubated with ^{14}C-AA in vitamin C transfer buffer and centrifuged after 5 and 10 minutes. The pellet was obtained, and the cells were lyzed using 2% SDS-Triton X-100 solution. Lysates were centrifuged, and ^{14}C-AA levels were measured by β-counter in scintillation cocktail fluid. Cell volume was normalized by comparing the amount of cytosolic protein extract from control with that from UVB-irradiated cells. Results are representative of more than three experiments, each performed in triplicate. (**b**) HaCaT cells were collected 2 and 4 hours after UVB irradiation (100 J/m^2), and then incubated with ^{14}C labeled-AA for 5 and 10 minutes in vitamin C transport buffer. The concentration of intracellular vitamin C was measured by high performance liquid chromatography (HPLC). The cell lysate was mixed with 10% metaphosphoric acid and then diluted with HPLC-grade distilled water. A 10 μl of sample was injected into the column C18 (GROM-SIL 120 ODS-5 ST, 5 μm). The vitamin C uptake was calculated with calibration curve. Intracellular vitamin C uptake was measured by HPLC at a UV-detection wavelength of 265 nm. Cell volume was normalized by comparing the amount of cytosolic protein extract from control with that from UVB-irradiated cells. Results are representative of more than three experiments, each performed in triplicate.

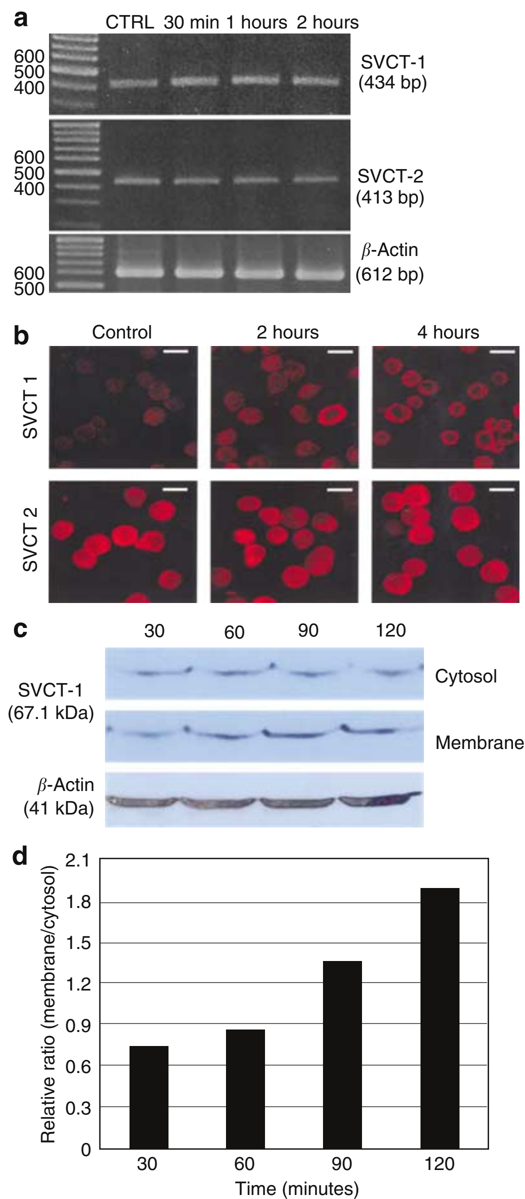

Figure 3. Post-transcriptional regulation of SVCT-1 expression in human keratinocyte cell line, HaCaT by UVB irradiation. (**a**) HaCaT cells were exposed to UVB (100 J/m^2) and then collected 0.5, 1, and 2 hours after UVB irradiation. Total RNA was extracted, and cDNA was synthesized for RT-PCR as described in Materials and Methods. Results are representative of three experiments. (**b**) After exposure to UVB (100 J/m^2), cells were collected and fixed. Cells were then resuspended in permeabilization buffer (0.15 M PBS containing 0.1% saponin) and immunostained using goat anti-human SVCT-1/-2 Ab (1 μg/ml) for 30 minutes at room temperature. Alexa Fluor 546-conjugated anti-goat Ab was used as secondary Ab. The distribution of SVCT proteins was investigated using confocal microscopy. Bar = 20 μm. Results are representative of three experiments. (**c**) The cytosolic and membrane protein of UVB-irradiated HaCaT cells were prepared 30, 60, 90, and 120 minutes after UVB irradiation (100 J/m^2) as described in Materials and Methods. An equal amount of protein (40 μg) was separated on 12% SDS-PAGE, transferred to the membrane, and blotted with anti-SVCT-1 antibody. Results are representative of three experiments. (**d**) Densitometry analysis; the images of membrane SVCT-1 to cytosol SVCT-1 was analyzed by analytical software (Scion Image Program, Frederick, MA).

Interestingly, there were no changes in SVCT-1/-2 mRNA transcript expression after UVB irradiation (Figure 3a).

There is a recent report on the movement of SVCT-1 from the cytosol to the membrane (Veedamali *et al.*, 2004). In addition, we already showed that there were no changes in SVCT-1/-2 mRNA expression. Therefore, we determined whether SVCT-1 and SVCT-2 migrate from the cytosol to the membrane to increase vitamin C uptake after UVB irradiation. As shown in Figure 3b, the translocation of SVCT-1, not

SVCT-2, was observed at 2 and 4 hours after UVB irradiation by confocal microscopy. In addition, we also obtained the similar results in primary epidermal keratinocytes (data not shown). To confirm the translocation of SVCT-1, we examined the changes in the amount of SVCT protein in the cytosol and membrane by immunoblotting. In accordance with the result obtained by confocal microcopy, SVCT-1 protein in the cytosol was decreased in a time-dependent manner, but membrane SVCT-1 was increased (Figure 3c and d). The membrane SVCT-1 reached a peak 90 minutes after UVB irradiation.

Regulation of UVB-induced chemokine production by vitamin C

To investigate the role of vitamin C in the regulation of UVB-induced chemokine expression, we determined the optical dose of vitamin C as an antioxidant. After UVB irradiation, intracellular ROS level was increased in HaCaT cells (Figure 3). However, enhanced ROS production by UVB irradiation was suppressed by post-treatment with vitamin C. Elevated vitamin C uptake by UVB irradiation is shown in Figure 2.

To determine the regulation of chemokine production by both UVB and increased vitamin C uptake, we screened the changes in the expression of mRNA transcripts of several chemokines. There were some increases of RANTES (regulated upon activation, normal T cell expressed and secreted) and MIP-1α, but IL-8 and MCP-1 mRNA expressions were markedly increased by UVB irradiation and suppressed by post-treatment with vitamin C (Figure 4a). Based on the results from RNase protection assay, we measured the concentrations of IL-8 and MCP-1 in the UVB-irradiated HaCaT cells by ELISA after incubation in the absence or presence of vitamin C. As shown in Figure 4c and d, UVB-induced IL-8 and MCP-1 production in the HaCaT cells was suppressed by post-treatment with vitamin C. We also obtained the similar results from the experiment using primary epidermal keratinocytes. In addition, UVB-induced chemokine production was suppressed by post-treatment with *N*-acetyl-L-cystein (data not shown).

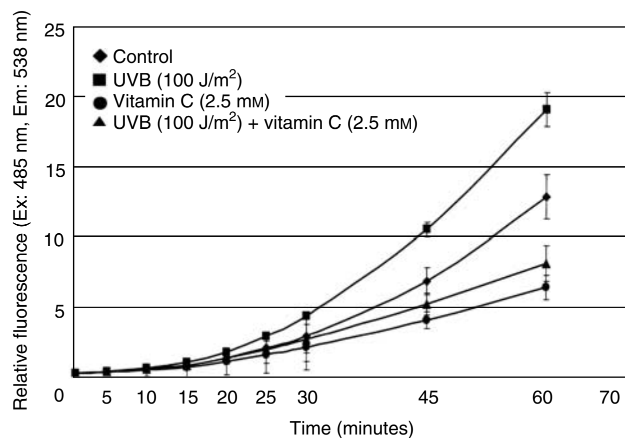

Figure 4. The role of vitamin C as an antioxidant in UVB-irradiated HaCaT. HaCaT cells (1×10^5/well) were post-treated with 2.5 mM vitamin C after exposure to 100 J/m^2 of UVB, and 50 μM DCFH-DA was added as a substrate for ROS. ROS level was measured by spectrofluorometry (excitation: 485 nm; emission: 538 nm). Results are representative of more than three experiments, each performed in triplicate. Data represent means $\pm$ SD. *P*-value of UVB and vitamin C treated group (▲) was less than 0.05, when it compared with UVB irradiated group (■).

The role of p38MAPK on UVB-induced chemokine production by vitamin C

Next, we examined which signals play an important role in the inhibition of chemokine by vitamin C. As shown in Figure 6a, when the p38MAPK inhibitor was added to UVB-irradiated HaCaT, IL-8, and MCP-1 production was markedly decreased. We also confirmed the same results on human primary keratinocyte (data not shown).

To investigate the changes of phosphorylated p38MAPK by UVB irradiation and vitamin C treatment, immunoblotting was performed. As shown in Figure 6c, the phosphorylation of p38MAPK was increased by UVB irradiation, but it was blocked by the immediate treatment of vitamin C after UVB irradiation. Taken together, UVB increases IL-8 and MCP-1 production via the activation of p38MAPK, but vitamin C down-regulates IL-8 and MCP-1 production via the suppression of UVB-induced p38MAPK activation.

DISCUSSION

UV radiation has diverse effects on the skin such as inflammation, photoaging, or skin cancer. Increased exposure to the UVB component of sunlight has been associated with high incidence of skin cancer (Niederkorn *et al.*, 1990; Tanabe *et al.*, 1993; Eton *et al.*, 1998; Weichenthal *et al.*, 2000). During the development of skin disease by UVB, ROS is known as an important mediator via alteration of DNA structure or induction of DNA damage (Masamitsu *et al.*, 2000). We hypothesized that antioxidants can be used as effective agents for the prevention of skin disease development by UVB. In other words, when the cells are exposed to UVB, they may need more antioxidant for scavenging UVB-induced ROS.

To test the hypothesis, we compared the amount of vitamin C in UVB-exposed skin keratinocytes with that in controls, since vitamin C is a well-known antioxidant. We found that vitamin C uptake was increased by UVB in a dose-dependent manner, as shown in Figure 2. Therefore, based on a report that inflammatory response following acute or chronic UVR exposure may contribute to skin carcinogenesis by oxidative stress mechanisms (Sander *et al.*, 2004), it is suggested that UVB-exposed skin keratinocytes may need more antioxidant for recovery from the damage by UVB-induced oxidative stress. Moreover, the antioxidant may inhibit carcinogenesis.

As mentioned above, the vitamin transport system through the plasma membrane is mediated by SVCT-1/2, a vitamin C-specific transporter. We focused on the changes in SVCT-1/-2 in UVB-irradiated HaCaT cells, since we found that vitamin C uptake was rapidly increased after UVB irradiation. Interestingly, in this study, it was demonstrated that vitamin C uptake was increased by UVB irradiation without any changes in SVCT-1/-2 mRNA transcript expression. However, we determined the redistribution of SVCT-1, not SVCT-2, from the

cytosol to the plasma membrane by confocal microscopy. In addition, we also found that the amount of SVCT-1 was decreased in the cytosol, but increased in the plasma membrane. Hence, it is indicated that the increase of vitamin C uptake is regulated not at the transcriptional level of SVCT-1, but at the post-transcriptional level of SVCT-1. According to a recent report by Veedamali *et al.* (2004) SVCT proteins reside in a heterogeneous population of intracellular organelles, and they are transported to the cell membrane. However, they did not show the redistribution of SVCT upon stimulation by external stimuli such as UVB. This study is probably the first one on the redistribution of SVCT-1 by UVB for rapid vitamin C uptake. In comparison to SVCT-1, there were no changes in the distribution of SVCT-2. It is generally accepted that SVCT-2 has a higher affinity for vitamin C than does SVCT-1. On the other hand, the capacity of SVCT-1 for the transportation of vitamin C is higher than that of SVCT-2. Therefore, the redistribution of SVCT-1, not SVCT-2, by UVB is an essential process for rapid uptake of vitamin C.

Vitamin C uptake was dependent on the dose of UVB, but not on the time of vitamin C treatment (Figure 2). This result demonstrates that the capacity of redistributed SVCT-1 for vitamin C uptake is saturated 2 hours after UVB irradiation. Therefore, the decreased vitamin C concentration at 4 hours represents the remainder of vitamin C after scavenging ROS, because we measured vitamin C as an ascorbate, not dehydroascorbic acid. It is inferred that the main function of vitamin C inside UVB-irradiated HaCaT cells is to mitigate inflammatory response induced by UVB because it has a great ability to scavenge reactive oxygen intermediates as an antioxidant.

To determine the effect of vitamin C on UVB-induced inflammation, we investigated the changes in chemokine production. Among the several kinds of chemokines, IL-8 and MCP-1 were predominantly increased. IL-8 induces keratinocyte proliferation (Steude *et al.*, 2002) and is involved in angiogenesis and growth of a variety of tumors such as melanoma (Huang *et al.*, 2002; Watanabe *et al.*, 2002). MCP-1 is a chemokine of the C-C or beta family that is chemotactic *in vitro* for monocytes, T cells and basophils. Its excessive production by keratinocytes has been implicated in psoriasis and other skin diseases. Hence, suppression of these chemokines is likely to play a significant role in the protection against UVB-induced skin inflammation and carcinogenesis.

We have already reported that vitamin C regulates the IL-18 production via the regulation of p38MAPK activation in our previous report (Cho *et al.*, 2003). Although there are several reports regarding the important role of p38MAPK on the production of IL-8 and MCP-1 (Hall *et al.*, 2005; Wong *et al.*, 2005; Zeng *et al.*, 2005), but still there is no report about the regulatory role of vitamin C on UVB-induced chemokine production via the regulation of p38MAPK activation (Figure 5). As shown in Figure 6, vitamin C effectively suppressed the phosphorylation of p38MAPK by UVB irradiation and the production of IL-8 and MCP-1. It suggests that vitamin C suppressed UVB-induced IL-8 and MCP-1 production via the activation of p38MAPK. In case of IL-8 production, it was not completely suppressed by the treatment of vitamin C, when it compared with the result from SB203580 treated HaCaT. Therefore, it thus indicates that more than one signal are involved in the production of IL-8 from HaCaT by UVB irradiation.

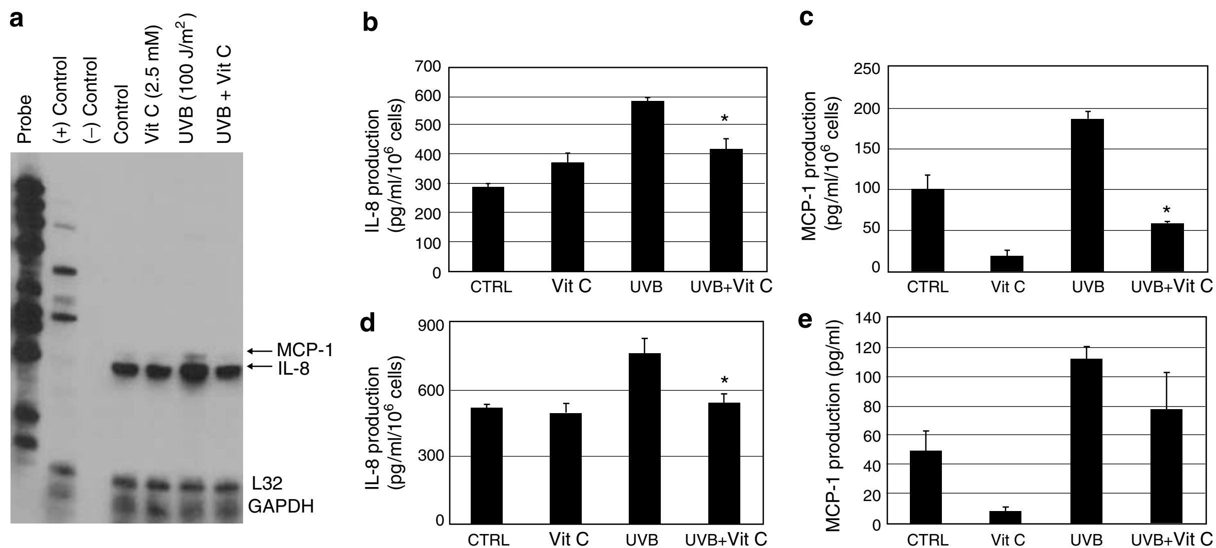

Figure 5. Regulatory effect of vitamin C on UVB-induced IL-8 and MCP-1 expression in human keratinocytes. (**a**) HaCaT cells were exposed to UVB (100 J/m^2) and post-treated with 2.5 mM of vitamin C for 4 hours. RNA was extracted, and RNase protection assay was carried out using hCK-5 multiprobe template set. RNA and ^{32}P-labeled mixture were separated on 5% acrylamide/8 M urea gel, dried, and exposed to X-ray film for 6 hours. Human and yeast RNA were used as positive and negative controls, respectively. (**b** and **d**) HaCaT and (**c** and **e**) primary epidermal keratinocytes were expose to UVB (100 J/m^2) and incubated for 12 hours in the presence or absence of 2.5 mM vitamin C. Culture supernatants were collected, and the changes in IL-8 and MCP-1 production were measured by ELISA. Results are representative of more than three experiments, each performed in triplicate. Data represent means $\pm$ SD. *P*-value of UVB and vitamin C treated group was less than 0.05, when it compared with UVB irradiated group.

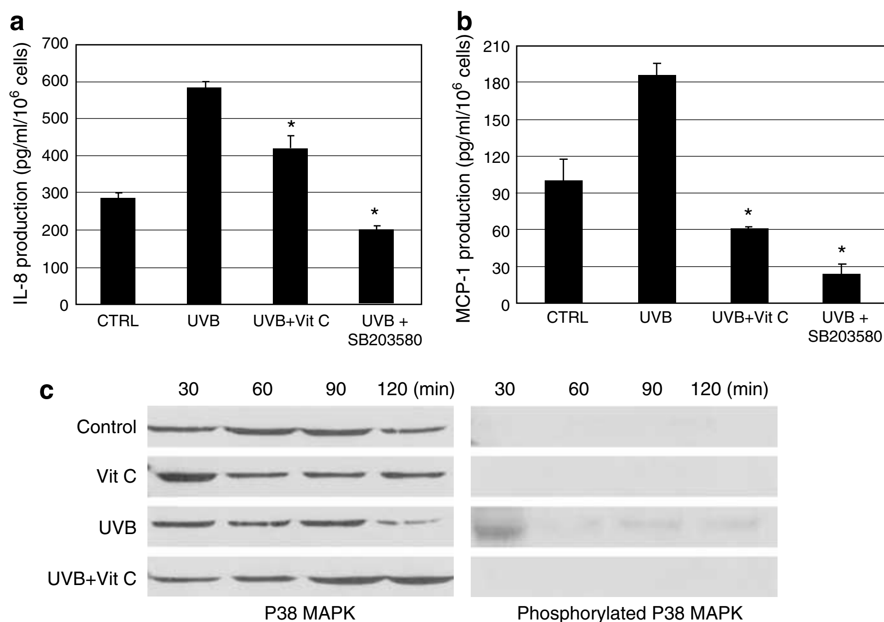

Figure 6. The role of p38MAPK on UVB-induced Chemokine Production by Vitamin C. (**a** and **b**) HaCaT cells were exposed to UVB (100 J/m^2) and post-treated with 2.5 mM of vitamin C and 10 μM of SB203580 for 12 hours. Culture supernatants were collected, and the changes in (**a**) IL-8 and (**b**) MCP-1 production were measured by ELISA. Results are representative of more than three experiments, each performed in triplicate. $P<0.05$ compared with corresponding values of control. (**c**) The cytosolic protein of UVB-irradiated HaCaT cells was prepared 30, 60, 90, and 120 minutes after UVB irradiation (100 J/m^2) as described in Materials and Methods. An equal amount of protein (40 μg) was separated on 12% SDS-PAGE, transferred to the membrane, and blotted with anti-p38 and phosphorylated-p38 antibody. Results are representative of three experiments. Data represent means $\pm$ SD. *P*-value of UVB and vitamin C treated group was less than 0.05, when it compared with UVB irradiated group.

Chemokines are regulated by the activation of NF-κB and activator protein-1 (Beak *et al.*, 2004). In fact, UVB-induced IL-8 and MCP-1 production is tightly regulated by activation of NF-κB (Simon *et al.*, 1994). In addition, the activation of NF-κB and activator protein-1 is significantly increased by UVB irradiation and regulated by intracellular redox state (Sen and Packer, 1996). Therefore, future studies on whether UVB-induced NF-κB and activator protein-1 activation is regulated by vitamin C will be required to determine whether the suppression of UVB-induced IL-8 and MCP-1 production by vitamin C is achieved via the regulation of NF-κB and activator protein-1.

In conclusion, this study is probably the first one on the translocation of SVCT-1 from the cytosol to the membrane by UVB irradiation. The translocation seems to be an essential process for the cells to protect themselves from the damage by UVB-induced ROS. In addition, increased uptake of vitamin C might effectively suppress inflammatory response via the downregulation of IL-8 and MCP-1 production.

MATERIALS AND METHODS

Cells and reagents

Human keratinocytes were obtained from the foreskin of healthy young male donors. Specimens were treated with dispase at 4°C for overnight and then epidermal cells were freshly isolated by 0.05% trypsin-EDTA solution for 15 minutes at 37°C. Purified human keratinocytes were cultured in a serum-free medium (KBM, BioWhittaker, Heidelberg, Germany) with full supplements (0.1 ng/ml human epidermal growth factor, 0.5 μg/ml hydrocortisone, 5 mg/ml insulin, 7.5 mg/ml bovine pituitary extract, 50 μg/ml gentamicin, 50 ng/ml amphotericin-B, and 0.15 mM calcium), according to the manufacturer's instructions. For our experiments, cells were derived from the fourth through the sixth passage grown as a monolayer to subconfluence. Contamination of the keratinocyte culture by fibroblasts was determined by flow cytometry using antihuman fibroblast mAb (clone ASO_2) throughout each experiment. Our institutional review board (Seoul National University College of Medicine) approved the study protocol and all experiments were conducted according to the Declaration of Helsinki Principles. Healthy individuals volunteered to participate after giving informed consent. The human keratinocyte cell line, HaCaT, was kindly provided by Dr Kyung Chan Park (Department of Dermatology, Seoul National University College of Medicine). This cell line in the log phase of growth was used for our experiments. Cells were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. The specific inhibitor of p38MAPK, SB203580, was purchased from Sigma (St Louis, MO) and used to investigate whether p38 MAPK plays a role in regulation of chemokine production by UVB irradiation and vitamin C treatment.

UVB irradiation

HaCaT and primary keratinocytes were plated on culture dish or 96-well plate for 24 hours and then exposed to UVB irradiation (290–320 nm) from a bank of lamps (Waldman, Schwenningen, Germany) placed 25 cm above the cells, after the removal of culture medium from the culture vessel. The irradiance of the lamps was measured with a calibrated photometer (Waldman). After exposure

to UVB, fresh culture medium was added, and the cells and media were harvested at the time point indicated.

Measurement of apoptosis

HaCaT cells were exposed to 50, 100, and 150 J/m^2, washed twice with cold phosphate-buffered saline (PBS), and then resuspended in 1 $\times$ binding buffer at a concentration of 1×10^6 cells/ml. One hundred microliters of the solution (1×10^5 cells) was transferred to a 5-ml culture tube. Five microliters of Annexin V-FITC was added, and cells were incubated at room temperature for 15 minutes in the dark with gentle vortexing. Four hundred microliters of 1 $\times$ binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) was then added to each tube. And 50 ng of propidium iodide (BD biosciences, CA) was added prior to analysis. The Annexin V-FITC apoptosis detection kit was purchased from Pharmingen (San Diego, CA). Flow cytometric analysis was performed using an Epics ALTRA (Coulter Co, Fullerton, CA) and data were anlaysed using the Expo32 program (Coulter Co, Fullerton, CA).

Measurement of intracellular ROS levels

HaCaT cells (1×10^5/well) were incubated in a 96-well plate for 6 hours and the culture medium was discarded, and exposed to 100 J/m^2 of UVB. Then the cells were simultaneously treated with 2.5 mM vitamin C and 50 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Eastman Kodak, Rochester, NY) was added to each well. The intracellular ROS levels were measured with a Cytofluor 2350 plate reader (Millipore, Bedford, MA) at an excitation wavelength of 485 nm and at an emission wavelength of 538 nm.

RNase protection assay

HaCaT cells (8×10^6) were exposed to 100 J/m^2 of UVB and cultured for 4 hours in the absence or presence of 2.5 mM of vitamin C. Total RNA was extracted from cells by using Easy-Blue (Intron Biotechnologies, Seoul, Korea). The hCK-5 multiprobe template for RNase protection assay was purchased from BD Biosciences-PharMingen (San Diego, CA), and RNase protection assay was performed according to the manufacturer's instructions. Briefly, 40 μg of total RNA was mixed with ^{32}P-labeled Riboquant kit. The unprotected RNA was digested using RNase T1/A mix. The digested RNA was purified and loaded on a 5% acrylamide/8 M urea gel. The dried radioactive gel was exposed to X-ray film and developed. Positive control (human RNA) was supplied by BD Multi-Probe Template Set (San Diego, CA).

ELISA assays

HaCaT cells (8×10^6) were exposed to 100 J/m^2 of UVB and cultured for 12 hours in the absence or presence of 2.5 mM of vitamin C or 10 μM of SB203580 (Sigma, MO) and the concentrations of IL-8 and MCP-1 in the culture supernatants were measured by ELISA. Human IL-8 and MCP-1 ELISA kits were purchased from R&D systems (Minneapolis, MN), and ELISA was performed according to the manufacturer's instructions. Briefly, culture supernatant was added to anti-IL-8 and MCP-1 Ab-coated wells. After 1-hour incubation at 37°C, the wells were washed four times with PBS-Tween-20 (pH 7.4). Alkaline phosphatase-conjugated anti-human IL-8 and MCP-1 Ab were added and incubated for another 1 hour. After the wells were washed four times with PBS-Tween-20, substrate solution was added, and incubated for another 1 hour. The relative absorbance was measured at 450 nm, and then IL-8 and MCP-1 concentrations were calculated with IL-8 and MCP-1 standard curves, respectively.

Measurement of Vitamin C Uptake

Cells (2×10^6) were exposed to UVB (290–320 nm) in a UVB-irradiation chamber and their ability to uptake vitamin C was examined using ^{14}C-labeled L-ascorbic acid. Briefly, cells were collected and resuspended in vitamin C transport buffer (15 mM HEPES, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, and 0.8 mM $MgCl_2$) at room temperature for 30 minutes. The uptake assay was performed at RT for 30 minutes in 500 μl of incubation buffer containing 0.5 mCi of ^{14}C-L-AA (Amersham Biosciences, specific activity 8.2 mCi/mmol) to a final concentration of 380 μM. The buffer was then removed. The cells were washed twice with ice-cold transport buffer and solubilized in 1.0 ml of lysis buffer composed of 0.5% SDS and 0.2 N NaOH. The 500 μl of cell lysate was transferred to a vial, and intracellular radioactivity was quantified by liquid scintillation spectrometry. The intracellular vitamin C content was also measured by HPLC at a UV-detection wavelength of 265 nm. The cell lysate was mixed with 10% metaphosphoric acid and incubated on ice for 30 minutes. The incubated cell lysate was centrifuged at 14,000 r.p.m. for 5 minutes, the supernatant was collected, and then an equal volume of distilled water was added (J.T. Baker; HPLC grade). A 10 μl of sample was injected into the column C18 (GROM-SIL 120 ODS-5 ST, 5 μm). The mobile phase consisted of 147 mM monochloroacetic acid, 112.5 mM NaOH, 1.6 mM Na_2-EDTA, 139 μM 1-octane sulfonic acid, and 5% methanol. The flow rate was 1 ml/min and the retention time of vitamin C was 2.333 minutes. The vitamin C uptake was calculated with the calibration curve.

Confocal microscopy

The distribution of SVCT in keratinocytes was assessed by confocal microscopy using goat anti-human SVCT-1 Ab and goat anti-human SVCT-2 Ab (Santa Cruz, CA). The 5×10^5 cells were cultured at 37°C in an atmosphere of 5% CO_2 for 24 hours. Cells were collected and resuspended in 15 ml of 1:1 mixture of 4% paraformaldehyde with FACS buffer (0.1% saponin, 0.05% BSA, and 0.02% sodium azide in PBS, pH 7.4). After cells were placed on ice for 15 minutes, they were washed, resuspended in 5 ml of permeabilization buffer (0.1% saponin and 0.05% sodium azide in PBS, pH 7.4), and incubated on ice for another 15 minutes. After addition of 1 μg/μl goat anti-human SVCT-1 Ab and goat anti-human SVCT-2 Ab, cells were cultured on ice for 30 minutes, and the sample was stained with Alexa Fluor 546-conjugated secondary Ab (Molecular Probes, CA).

RT-PCR

To examine the changes in SVCT-1/-2 mRNA expression, RT-PCR was performed. Briefly, cells were harvested for 30 minutes, 1 hour, and 2 hours after UVB irradiation and washed three times with PBS. Total RNA was isolated from 8×10^6 cultured cells using TRIZOL, and cDNA was made using AMV RT. Oligo(dT)$_{15}$ was used as a primer. Thereafter, cDNA was amplified using the following primers (SVCT-1: (F) 5′-AGA TGA GGG CCC AAG AGG A-3′, (R) 5′-TGT TCA GGG GCA GAC TCC A, product = 434 bp; SVCT-2: (F) 5′-GGC TGG AAG TTC AAC AGA-3′, (R) 5′-GTA GTG ATT CCC ACA CAG A-3′, product = 400 bp). Cycling conditions for SVCT-1 and SVCT-2 were 1 minute each at 94, 65, and 72°C for 40 cycles. PCR products were separated by electrophoresis on 1.5% agarose gels and visualized by staining with ethidium bromide.

Western blot analysis

HaCaT cells were exposed to UVB (100 J/m^2), and the cells were incubated for 30, 60, 90, and 120 minutes. The cells were collected and divided into two: one for the preparation of cytosolic protein and the other one for the membrane fraction protein. For isolation of cytosolic protein, cells were resuspended in lysis buffer (20 mM HEPES and 2 mM EDTA) and subjected to repeated freezing and thawing. After centrifugation at 4°C at 12,500 r.p.m. for 1 hour, the supernatant was collected and used as cytosolic protein. For isolation of membrane protein, the cells pellet was resuspended in ice-cold Dounce buffer (10 mM Tris-Cl, pH 7.6, 0.5 mM $MgCl_2$) with protease inhibitors and was left alone at 4°C for 10 minutes. The cells were homogenized by Dounce homogenizer, and tonicity restoration buffer (10 mM Tris-Cl, pH 7.6, 0.5 mM $MgCl_2$, 0.6 M NaCl) containing protease inhibitors was added to the homogenized cells; its final concentration was 0.15 M NaCl (the suspension:tonicity restoration buffer = 1:3). The suspension was centrifuged at 4°C at 1,500 r.p.m. for 5 minutes. The supernatant was obtained, and 0.5 M EDTA (pH 7.6) was added to the supernatant; its final concentration was 5 mM. The mixture was centrifuged at 4°C at 125,000 r.p.m. for 45 minutes. The supernatant was removed, and the membrane pellet was resuspended in Triton X-100 lysis buffer (50 mM Tris-Cl, pH 7.6, 300 mM NaCl, and 0.5% Triton X-100) with protease inhibitors. After the pellet was dissolved by continuous gentle vortexing at 4°C for 30–45 minutes, the mixture was centrifuged at 4°C at 10,000 r.p.m. for 15 minutes and the supernatant was obtained. The protein concentration was measured using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). An equal amount of protein (50 μg) was dissolved in a 12% polyacrylamide-SDS gel with 100 V for 8 hours and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% nonfat milk, washed with 0.1% Tween 20-PBS for 1 hour, and then exposed to primary antibody at RT for 1 hour. Primary antibodies were diluted 1:100 (goat anti-SVCT-1 and SVCT-2 antibody, Santa Cruz, CA), 1:1,000 (rabbit anti phospho-p38 MAPK antibody, Cell signaling, MA), and 1:2,000 (mouse anti-p38 MAPK antibody, Cell signaling, MA) in 0.1% Tween-20-PBS. After the blots were washed, they were exposed to biotin-conjugated secondary antibody at RT for 1 hour. The membrane was then washed, incubated with a 1:5,000 dilution of streptavidin-horseradish peroxidase, and the immunoreactive proteins were visualized with the enhanced chemiluminescence detection system (Amersham). The analysis of the images of membrane SVCT-1 to cytosol SVCT-1 was performed by using, analytical software (Scion Image Program, Frederic, MA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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