Effect of Persimmon Leaf Extract on Utraviolet B-induced Inflammation in HaCaT Keratinocytes and Mice

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Ultraviolet B (UVB) irradiation induces skin damage and inflammation. Persimmon leaf extract (PLE) has traditionally been used for the treatment of acute and chronic diseases in Asia, but there have been no reports on its UVB-protective effects. Thus, protective effects of PLE against UVB-induced inflammations in HaCaT keratinocytes and mice were investigated. In an *in vitro* study using HaCaT keratinocytes, UVB irradiation decreased cell viability and increased chemokine production. PLE recovered cell viability and suppressed UVB-induced production of monocyte chemotactic protein-1 (CCL2) and cutaneous T cell-attracting chemokine (CCL27). Moreover, oral administration of PLE to UVB-irradiated mice resulted in significant suppression of skin damage. Histological analyses revealed that infiltration of inflammatory cells, especially degranulated mast cells, thickening of the epidermis, and hyperplasia were significantly reduced. PLE treatment suppressed UVB-induced CCL2 and CCL27 expressions in the skin. These results suggest PLE has potential as an effective material for protection against UVB-induced skin inflammation.

Key words: anti-inflammation, HaCaT keratinocytes, mice, persimmon leaf extract, ultraviolet B

One of the most significant risk factors associated with the development of skin disease is exposure to ultraviolet (UV) radiation from the sun. In particular, UVB (290~ 320 nm) irradiation can activate inflammatory pathways, leading to skin damage [Weichenthal *et al*., 2000]. It is well known that UVB induces inflammation in skin by the transcription and release of cytokines and chemokines from skin keratinocytes [Fisher *et al*., 1997; Di Girolamo *et al*., 2006; Shimauchi *et al*., 2008].

Chemokines are small proteins that mediate attraction, activation, and differentiation of leukocytes. They also regulate cell trafficking of various types of leukocytes through interactions with a subset of seven-transmembrane, G protein-coupled receptors [Rossi and Zlotnik, 2000]. Among chemokines, cutaneous T cell-attracting chemokine (CCL27) is a skin-specific CC chemokine that is produced constitutively by keratinocytes and is highly upregulated in inflammatory skin conditions upon UVB exposure [Meller *et al*., 2005]. Monocyte chemotactic protein-1 (CCL2) is known as a member of the proinflammatory CC chemokine superfamily and plays a critical role in the recruitment of monocytes and lymphocytes during the inflammatory response in response to UVB irradiation [Kang *et al*., 2007]. Therefore, agents that regulate these inflammatory molecules could provide beneficial anti-inflammatory effects. Current treatments for inflammation include aspirin, nonsteriodal anti-inflammatory drugs, and dexamethasone. However, these compounds tend to have adverse side effects, which could be ameliorated by the use of effective botanical alternatives.

Persimmon (*Diospyros kaki*), widely distributed in East Asia, is a traditional plant medicine for the treatment of hypertension, angina, and internal hemorrhaging [Mallavadhani *et al*., 1998]. Recently, researchers reported that persimmon leaf extract (PLE) as well as astragalin from PLE inhibit the development of atopic dermatitis in NC/Nga mice [Kotani *et al*., 2000; Matsumoto *et al*., 2002]. However, there have been no reports on their UVB-protective effects. Therefore, the

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purpose of the present study was to investigate the protective effects of PLE against UVB-induced inflammation in HaCaT keratinocytes and mice.

Materials and Methods

Reagents. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO-BRL (Invitrogen Co., Grand Island, NY). Penicillin-Streptomycin, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT), Congo red, and toluidine blue were purchased from Sigma (St. Louis, MO). CCL2 and CCL27 ELISA kits for human and mouse were obtained from R&D Systems (Minneapolis, MN). Anti-mouse CCL2 and anti-mouse CCL27 monoclonal antibody (mAb) conjugated with PE were obtained from BD Biosciences (San Jose, CA). Anti-FcεRIα mAb was purchased from Kyokuto Pharmaceutical Industry Co Ltd (Tokyo, Japan). Other chemicals of reagent grade were purchased from Sigma.

Persimmon leaf material and extraction. Persimmon (*D. kaki*) leaf materials were collected on 20 Jun 2010 from Bugui-myen and Jeongcheon-myen, Jinan-gun (Jeonju, Korea). The plant was identified and authenticated by Prof. Hong Jun Kim at the College of Oriental Medicine, Woosuk University. A voucher specimen was deposited in the authors laboratory (Seon Il Jang). Persimmon leaf of *D. kaki* was steeped in boiling distilled water for 5 min and then filtered through a nylon mesh (pore size, 40 μm). After centrifugation at 5,000 rpm for 30 min at 4°C, the supernatant of PLE was freeze-dried and kept at −20 °C. The level of contaminated endotoxin in persimmon leaf was less than 1 ng/mL.

Cell line and culture. HaCaT human keratinocytes were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured with DMEM containing 10% heat-inactivated FBS, penicillin G (100 IU/mL), and streptomycin (100 mg/mL), followed by incubation at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Medium was changed every 2 days. Cells were cultured to 70-80% confluence in a round plate (10 cm in diameter) and expanded by subculturing into four plates. When the cell population reached 80% confluence, cells were seeded in 24-well plates and incubated for 24 h.

UV irradiation and PLE treatments. HaCaT cells were plated at a concentration of 3×10^4 cells per well in 24-well plates containing DMEM supplemented with 10% FBS and antibiotics and then maintained in a cell culture incubator. After 24 h, cells were directly irradiated with 25 mJ/cm² of UVB (0.71-0.74 mW/cm²) using GL20SE UVB lamps (emission peak 306 nm; Sankyo

Denki Co., Tokyo, Japan). To prevent light absorption by the culture medium, the medium was removed prior to irradiation and replaced with just enough phosphate-buffered saline (PBS) to cover the cells. After UVB exposure, cells were cultured in serum-free medium for 24 h with or without PLE.

MTT assay. MTT was used as a cell viability indicator based on its mitochondrial-dependent reduction to formazan. HaCaT cells were plated at 3×10^4 cells/100 μL into 96-well plates and then incubated. After 24 h, 100 mL of MTT stock solution (50 μg/mL) was added to the plate, followed by incubation for additional 5 h. The medium was then removed, and any formazan present was dissolved in 100 μL of dimethyl sulfoxide. Absorbance at 570 nm was measured using an ELISA reader (EMax®, Molecular Devices, Sunnyvale, CA). The ratio of optical density at 570 nm of each reagent-treated well to the corresponding control was calculated as a percentage.

Measurement of chemokines from culture supernatants. Culture supernatants from UVB-irradiated and non-irradiated (control) cells were carefully harvested after 24 h for chemokine assays and then stored at −70°C until use. Levels of CCL2 and CCL27 were measured using the appropriate ELISA kits according to the manufacturer's protocols (R&D Systems). Absorbance at 450 nm was measured using an ELISA plate reader.

Animals. Balb/c 6-week-old mice were purchased from Central Lab. Inc. (Seoul, Korea), the Korean branch of Charles River Japan (Kanagawa, Japan). Mice were maintained in an environmentally-controlled rearing system and used for experiments when 8-week-old. All experiments were performed in accordance with Jeonju University Institutional Animal Care and Use Committee guidelines.

PLE administration and UVB irradiation. The control group and UVB-irradiated only group were orally administrated 200 μL of saline daily for 20 days. The PLE-treated group was orally administrated with a single dose of PLE (250 mg/kg/day) for 20 days. The positive control group was treated with vitamin C (25 mg/kg of L-ascorbic acid). On days 15, 17, and 19, the dorsal skin of each Balb/c mouse was directly exposed to 250 mJ/cm² of UVB irradiation (0.71-0.74 mW/cm²) using GL20SE UVB lamps (emission peak 306 nm; Sankyo Denki Co., Tokyo, Japan). Each experimental group consisted of five mice. Dorsal skin for the observation of histology and immunological phenomena was separated 24 h after the last irradiation. Sera for chemokine evaluation were also isolated from blood 24 h after the last irradiation.

Histological examination. Dorsal tissues were fixed with 4% paraformaldehyde and embedded in paraffin, after which 5 μm-thin sections were made on silane-
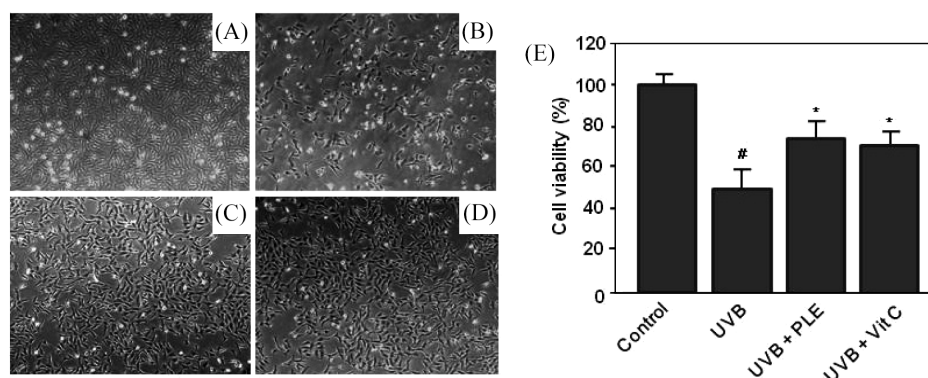
Fig. 1. Effect of persimmon leaf extract (PLE) on UVB-induced cytotoxicity in HaCaT cells. Cells were pretreated for 2 h with or without PLE (250 μg/mL) or vitamin C (2.5 mM) and then irradiated with UVB (25 mJ/cm²). After 24 h, cell viability was determined by MTT assay. Microscopic findings of A (Normal control), B (UVB-irradiated control), C (PLE plus UVB irradiation), D (vitamin C plus UVB irradiation), and E (cell viability by MTT assay). Values are shown as means±SD from three independent experiments. $^{\#}p<0.001$ indicates significant difference with the control group. $*p<0.05$ indicates significant difference with the UVB-irradiated only group.

coated micro slides (Muto-glass, Tokyo, Japan). The de-paraffinized skin sections were stained with hematoxylin & eosin (H&E), Congo red or toluidine blue and then examined by light microscopy (Olympus, Tokyo, Japan) to assess histological changes and leukocyte infiltration.

Immunohistochemical analysis. The de-paraffinized skin sections were stained with anti-mouse CCL2 and CCL27 antibodies. Sections for immunohistochemistry were de-paraffinized with xylene, rinsed with distilled water, and then again with PBS. The non-specific antigen-antibody reactions were inhibited by treatment for 1 h with 10% normal goat serum. The sections were reacted overnight at 4°C in diluted solutions of each primary antibody, such as purified rat monoclonal antibodies against anti-mouse CCL2 and CCL27. The sections were then rinsed with distilled water and PBS and then covered with a cover slip. Fluorescence microscopy (Olympus) was then performed to assess the expression levels.

Measurement of chemokines from serum. Serum samples were collected by portal vein extraction 24 h after the last irradiation. In brief, blood samples were allowed to clot for 30 min at room temperature and then centrifuged for 20 min at 1,000 g. Serum was carefully collected and stored at −90°C until use. Levels of CCL2 and CCL27 were measured using the appropriate ELISA kits according to the manufacturer's protocols (R&D Systems). Absorbance was measured at 450 nm using an ELISA reader.

Statistical analysis. Differences in data among the groups were analyzed by one-way ANOVA, and all values were expressed as mean±SD or SE. Differences between the groups were considered to be significant at $p<0.05$.

Results

Effect of PLE on viability of UVB-irradiated HaCaT cells. The effect of PLE on UVB irradiation was tested in HaCaT cells. Cell morphology and viability were evaluated by light microscopy and MTT assay under various conditions (Fig. 1). When cultures were incubated for 24 h after UVB irradiation, UVB-induced toxicity was increased compared to untreated cells (Fig. 1B). Cell viability decreased in response to 25 mJ/cm² of UVB irradiation after 24 h ($p<0.001$) (Fig. 1E). However, cells treated with PLE or vitamin C demonstrated significantly higher cell viability than those of the UVB-irradiated only group ($p<0.05$).

Effect of PLE on chemokine production in UVB-irradiated HaCaT cells. To investigate the effect of PLE treatment on chemokine production related to leukocyte infiltration, HaCaT cells were cultured with PLE or vitamin C, and the levels of CCL2 and CCL27 were measured by ELISA in the culture supernatants of HaCaT cells. Production of CCL2 and CCL27 was significantly increased in the UVB-irradiated only group compared to that of the UVB-untreated control group (Fig. 2). In contrast, CCL2 and CCL27 levels were significantly reduced in cells treated with PLE or vitamin C compared to those of the UVB-irradiated only group ($p<0.05$). This result demonstrates that PLE effectively suppressed chemokine production related to leukocyte infiltration in HaCaT cells.

Effect of PLE on histological changes in UVB-irradiated skin tissues. To investigate whether or not PLE could be effective for UVB irradiation *in vivo*, H&E staining was performed and the thickness of dorsal skins in Balb/c mice specimens was measured. UVB exposure
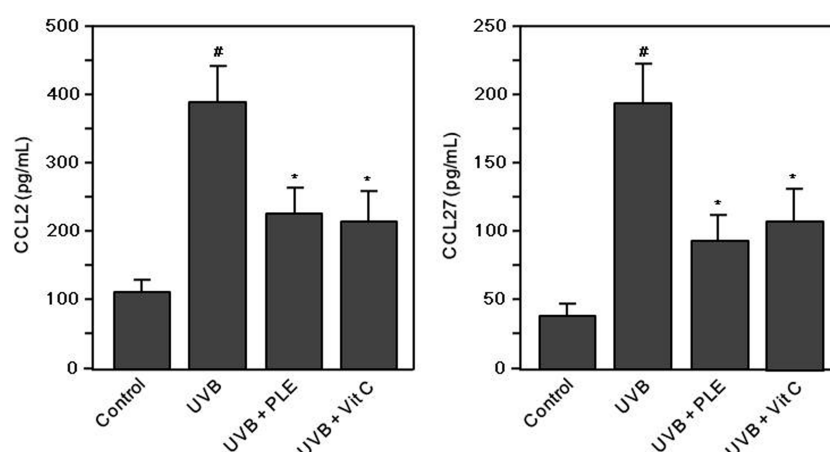
Fig. 2. Inhibitory effect of PLE on UVB-induced chemokine production in HaCaT cells. Cells were pretreated for 2 h with or without PLE (250 μg/mL) or vitamin C (2.5 mM) and then irradiated with UVB (25 mJ/cm^2). After 24 h, production of CCL2 and CCL27 in supernatants was measured by ELISA. Values are shown as means±SD of three independent experiments. $^{#}p<0.001$ indicates significant difference with the control group. $^{*}p<0.05$ indicate significant difference with the only UVB-irradiated group.
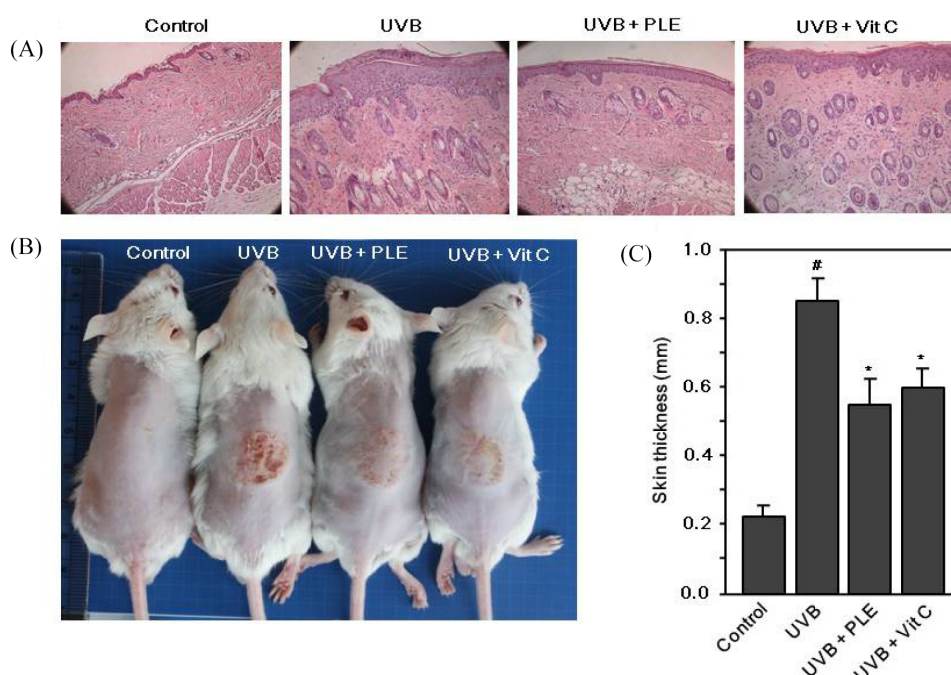

Fig. 3. Effect of PLE administration on UVB-induced histological changes and skin thickness. Oral treatment with PLE (250 mg/kg, body weight (BW) or vitamin C (25 mg/kg, BW) was carried out for 20 days in mice. On days 15, 17, and 19, mouse skin was directly exposed to 200 mJ/cm^2 of UVB irradiation from the vertical position to the back. (A) Skin tissues (5 μm) were stained with H&E. Original magnification was ×200 for all photos. (B) Morphological changes in the skin upon PLE administration in UV-irradiated Balb/c mice. (C) Skin thickness in UV-irradiated Balb/c mice. Values are shown as means±SE of the changes in skin thickness and in five mice of each group. $^{#}p<0.001$ indicates significant difference with the control group. $^{*}p<0.05$ indicates significant difference with the UVB-irradiated only group.

induced edema, erythema, and thickening of the dorsal skin in untreated mice (Fig. 3). UVB-irradiated mouse skin exhibited hyperplasia and acanthosis (thickening) in the epidermis and dilated blood vessels in the dermis compared to UVB-untreated control animals (Fig. 3A). In contrast, UVB-induced histological and morphological changes were significantly reduced in mice treated with PLE or vitamin C. In particular, UVB-induced skin thickness was significantly reduced in PLE- and vitamin C-treated specimens compared to that of the UVB-irradiated only group ($p<0.05$). These results suggest that PLE exerts effective protection against UVB-induced skin damage.
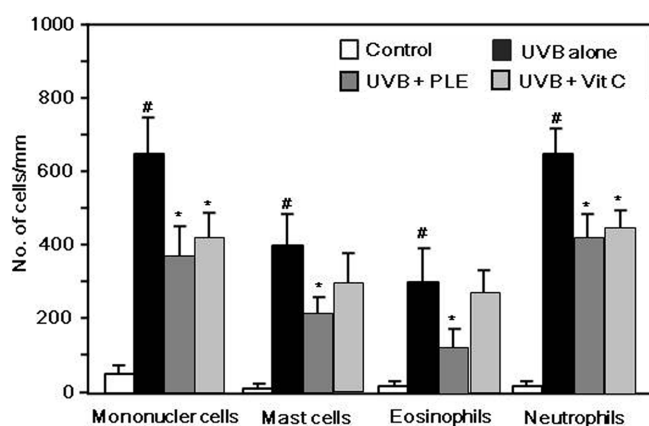
Fig. 4. Effect of PLE administration on UVB-induced leukocyte infiltration in Balb/c mice. Oral treatment with PLE (250 mg/kg, BW) or vitamin C (25 mg/kg, BW) was carried out in UV-irradiated Balb/c mice for 20 days. On days 15, 17, and 19, mouse skin was directly exposed to 200 mJ/cm^2 of UVB irradiation from the vertical position to the back. Skin tissues (5 μm) were stained with H&E or Congo red. Leukocyte infiltrations were measured at a ×400 microscopic view. Values are shown as means±SE of the changes in leukocyte infiltration in five mice of each group. $^{#}p<0.001$ indicates significant difference with the control group. $^{*}p<0.05$ indicates significant difference with the UVB-irradiated only group.

Effect of PLE on UVB-induced leukocyte infiltration and chemokine production. To investigate the effect of PLE treatment on leukocyte infiltration in UVB-irradiated mice, skin tissues were stained with H&E, Congo red or toluidine blue. Dorsal skin sections in the UVB-irradiated only group showed significantly increased infiltration of leukocytes, including mononuclear cells, mast cells, eosinophils, and neutrophils ($p<0.001$) (Fig. 4). In contrast, sections of skin in the PLE- or vitamin C-treated group showed significantly diminished leukocyte infiltration into the epidermis and dermis ($p<0.05$). Finally, to investigate the inhibitory effect of PLE on the expressions of CCL2 and CCL27, immunohistochemical staining of skin specimens was performed. CCL2 and CCL27 expressions in the skin were increased in the UVB-irradiated only group (Fig. 5A). However, they were markedly decreased upon oral administration of PLE or vitamin C. The effect of PLE on the serum levels of CCL2 and CCL27 was also analyzed. Similar to the expressions of CCL2 and CCL27 in the skin, oral administration of PLE or vitamin C markedly reduced UVB-induced CCL2 and CCL27 production in the sera (Fig. 5B). These results demonstrate that PLE has an inhibitory effect on UVB-induced leukocyte infiltration by inhibiting CCL2 and CCL27 production.

Discussion

In Korea and Japan , persimmon leaf tea has been traditionally believed to be a health-promoting beverage, similar to the green tea [Han et al., 2002]. Until now, studies of PLE has focused on natural compounds including vitamin C, polyphenols, flavonoids, hydrocarbons, triterpene, and steroids [Chen et al., 2009; Kawakami et al., 2010]. In a hot water extract of persimmon leaves, 0.59% of kaempferol-3-glucoside (astragalin), one of the flavonoids, was found [Kotani et al., 2000]. Recently, Kotani et al. [2000] and Matsumoto et al. [2002] reported strong inhibitory effects of kaempferol-3-glucoside on the histamine release and the atopic dermatitis in 250 μg/mL (in vitro) and 250 mg/kg (in vivo) nontoxic concentrations of PLE. However, there has been no report on the preventative effect of PLE against skin damage by UVB irradiation.

Agents that regulate these inflammatory molecules could have beneficial anti-inflammatory effects. Thus, considerable effort is being made to screen potential plants that can reduce UVB-induced human skin inflammation. Black tea and green tea [Record and Dreosti, 1998], ginger [Guahk et al., 2010], and broccoli [Shibata et al., 2009] have been reported to prevent UVB-induced skin disorders in mice. The present study was designed to compare the protective effects of PLE against UVB radiation-induced damage to HaCaT cells and to the skin of Balb/c mice. Here, PLE suppression of UVB-induced cytotoxicity and chemokine production in HaCaT keratinocytes was demonstrated. These in vitro data together with the in vivo results obtained from Balb/c mice indicate that PLE has the potential efficacy to ameliorate UVB-induced skin damage.

Exposure of skin to UVB irradiation results in a variety of biological effects, including inflammation, sunburn cell formation, immunologic alterations, and photo-aging [Ito et al., 2003; Yaar and Gilchrest, 2007]. UVB radiation critically damages cellular macromolecules and induces the formation of reactive oxygen species (ROS) [Fuchs, 1992]. The epidermal antioxidant defense system combats ROS-induced oxidative damage; however, antioxidant levels declined following UVB exposure [Shindo et al, 1993]. An interesting strategy for improving photo-protection is the support of endogenous antioxidant system of the skin with exogenous supplementation [Steenvoorden and van Henegouwen, 1997].

Pretreatment of cells with FLDK-P70 from persimmon leaf alleviates H$_2$O$_2$-induced cell injury by improving redox imbalance, as evidenced by the reversal of the
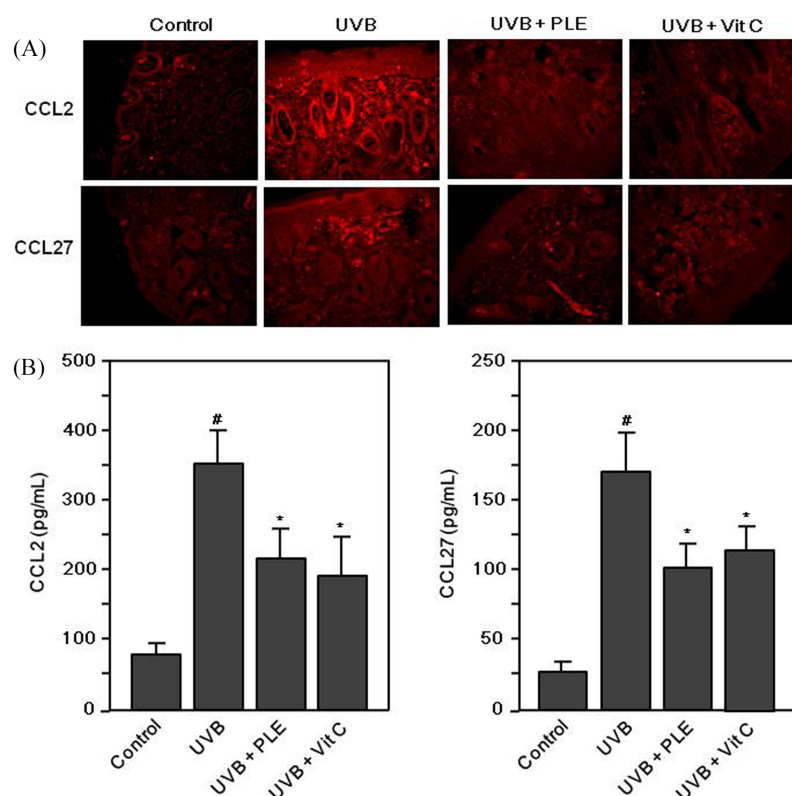
Fig. 5. Inhibitory effects of PLE administration on UVB-induced chemokine expression and production in Balb/c mice. Oral treatment with PLE (250 mg/kg, BW) or vitamin C (25 mg/kg, BW) was carried out for 20 days. On days 15, 17, and 19, mouse skin was directly exposed to 200 mJ/cm^2 of UVB irradiation from the vertical position to the back. One day after UVB irradiation, tissue samples were prepared from the back skins. (A) Staining for chemokine expression was performed by immunochemistry with anti-mouse CCL2 or CCL27 antibody. Chemokine expression levels were measured at a ×400 microscopic view. (B) Chemokine levels in the sera were measured using a CCL27 or monotype chemoattractant protein 1 (MCP-1) ELISA assay kit. Values are shown as means±SE of the changes in chemokine levels in five mice of each group. $^{\#}p<0.001$ indicates significant difference with the control group. $^{*}p<0.05$ indicates significant difference with the UVB-irradiated only group.

decline in intracellular endogenous antioxidants [Bei et al., 2005]. In a previous study, we reported that PLE has potent antioxidant activity (DPPH- and NO-radical scavenging activity and superoxide dismutase-like activity) [Jeong et al., 2010]. Vitamin C is one of the most frequently investigated antioxidants, which protects the cells from the ROS-mediated damage by UVB irradiation [Reddy and Bhat, 1999]. Therefore, in this study, we used vitamin C with positive control against UVB radiation-induced damage to HaCaT cells and to the skin of Balb/c mice. In this study, we found that PLE had protective effects against UVB-induced keratinocyte injury, similar to vitamin C (Fig. 1).

Infiltration of leukocytes, which is believed to be initiated by locally generated chemotactic stimuli and often accompanied by simultaneous increases in vascular permeability and blood flow, is an important event during acute inflammation in the skin [Issekutz and Movat, 1982]. In this study, UVB irradiation significantly increased infiltration of leukocytes, including mononuclear cells,

mast cells, eosinophils, and neutrophils. However, PLE administration significantly diminished leukocyte infiltration into the epidermis and dermis (Fig. 4).

Keratinocytes synthesize many chemokines, including members of the CC and CXC subfamilies. They also express certain chemokine receptors that mediate the inflammatory or immune response by attracting various kinds of leukocytes. Keratinocytes respond to the major changes in inflammation and immunomodulation observed after UVB exposure via the UVB-induced release of inflammatory mediators such as cytokines and chemokines [Pupe et al., 2002; Kang et al., 2007; Storey et al., 2007]. Immigration of Langerhans cell (LC) precursors into the epidermis induces keratinocytes to secrete CCL2, which constitutively recruits dendritic cells and LCs to the skin [Nakamura et al., 1995]. CCL2 production is increased under inflammatory conditions in UVB-irradiated skin [Xia et al., 2010]. CCL27 chemokine binds specifically to CCR10, which is expressed by epidermal LCs [Soler et al., 2003; Meller et al., 2005]. In this study, we found that

PLE administration inhibited UVB-induced CCL2 and CCL27 production in HaCaT keratinocytes (Fig. 2). In addition, we showed that PLE administration suppressed UVB-induced leukocyte infiltration by inhibiting CCL2 and CCL27 production in Balb/c mice (Fig. 4 and 5). In fact, UVB-induced CCL2 and CCL27 production is tightly regulated by activation of nuclear factor kappa B (NF-κB) [Simon *et al.*, 1994]. In addition, the activation of NF-κB and activator protein-1 (AP-1) is significantly increased by UVB irradiation and regulated by the intracellular redox state [Sen and Packer, 1996]. Therefore, further studies are required to fully understand the protective effects of PLE against skin damage by UVB irradiation in molecular level. Additionally, studies on the pharmacological actions of major flavonol such as astragalin from PLE in relation to skin specific cytokines and chemokines, as well as interactions between PLE and UVB protection system are needed.

In summary, our group is the first, to the best of our knowledge, to provide evidence that oral administration of PLE can improve UVB-induced skin inflammation in mice by supressing CCL2 and CCL27 chemokine production. Altogether, results of the present study suggest that PLE administration may be a beneficial approach to the treatment of UVB-induced skin damage.

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