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Anti-Inflammatory Effect of *Ixeris dentata* on Ultraviolet B-Induced HaCaT Keratinocytes

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Abstract – Human skin is the first line of defense for the protection of the internal organs of the body from different stimuli. Ultraviolet B (UVB) irradiation induces skin damage and inflammation through the secretion of various cytokines, which are immune regulators produced by cells. To prevent the initiation of skin inflammation, keratinocytes that have been irreversibly damaged by radiation must be removed through the apoptotic mechanism. *Ixeris dentata* (family: Asteraceae) is a perennial medicinal herb indigenous to Korea. It has been used in Korea, China, and Japan to treat in digestion, pneumonia, diabetes, hepatitis, and tumors. To gain insight into the anti-inflammatory effects of *I. dentata*, we examined its influence on UVB-induced pro-inflammatory cytokine production in human keratinocytes (HaCaT cells), by observing cells that were stimulated with UVB in the presence or absence of *I. dentata*. In the present study, pro-inflammatory cytokine production was determined by performing enzyme-linked immunosorbent assay, reverse transcription polymerase chain reaction, and western blot analysis to measure the activation of mitogen-activated protein kinase (MAPKs). *I. dentata* inhibited UVB-induced production of the pro-inflammatory cytokine interleukin (IL)-6 in a dose-dependent manner. Further, *I. dentata* inhibited the UVB-induced expression of cyclooxygenase (COX)-2. Furthermore, *I. dentata* inhibited the phosphorylation of c-Jun NH2-terminal kinase and p38 MAPKs, suggesting that it inhibits the secretion of the pro-inflammatory cytokines IL-6 and IL-8, and COX-2 expression, by blocking MAPK phosphorylation. These results suggest that *I. dentate* can potentially protect against UVB-induced skin inflammation.
Keywords – *Ixeris dentata*, ultraviolet B, human keratinocytes

Introduction

Exposure to excessive sunlight is an important etiologic factor in the development of acute inflammation, characterized by erythema, edema, and immunosuppression, and thus consequently linked to the progression of skin cancer (Granstein and Matsui 2004; Matsumura and Ananthaswamy 2004). Ultraviolet B (UVB) is a well-known major risk factor for the development of acute inflammation as well as non-melanoma skin cancer in the epidermis (De Fabo *et al*., 2004; Ramos *et al*., 2004). Accumulating data indicate that UVB exerts its detrimental effect mainly through the induction of direct DNA damage or the production of reactive oxygen species (ROS) (de Gruijl 2002; Kulms *et al*., 2002; Heck *et al*., 2003). Direct DNA damage and ROS often trigger signaling pathways such as those of the mitogen-activated protein kinases (MAPKs), which are involved in cell proliferation and survival (Rhee 1999; Torres and Forman 2003). The MAPKs are a family of proline-directed Ser/Thr kinases composed of extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38 MAPK. Recent studies have shown that the activation of ERK, JNK, and p38 MAPK is tightly correlated with acute inflammation and development of skin cancer through increased expression of cyclooxygenase-2 (COX-2) (Chen *et al*., 2001; Lin *et al*., 2004; Mahns *et al*., 2004).

Keratinocytes are the major target of UVB damage and play a central role in the inflammatory and immunomodulatory changes observed after UV exposure, at least partly via the UV-induced release of cytokines (Takashima and Bergstresser 1996) and COX products such as

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prostaglandin E2 (Grewe *et al*., 1993).

Interleukin-6 (IL-6) is an interleukin that acts as both a pro-inflammatory and an anti-inflammatory cytokine. IL-6 is secreted by T cells and macrophages to stimulate the immune response to trauma, especially burns or other tissue damage, leading to inflammation (Muraguchi *et al*., 1981; Naka *et al*., 2002). Additionally, IL-6 modulates a variety of physiological events that occur in the nervous system, endocrine system, and bone metabolism (Naka *et al*., 2002; Kamimura *et al*., 2003).

COX is a bifunctional enzyme that first catalyzes the addition of 2 molecules of oxygen to arachidonic acid to form the hydroperoxide prostaglandin G2, and then reduces the hydroperoxide to the corresponding alcohol, prostaglandin H2, via peroxidase activity (Smith and Song 2002). COX has 2 isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in most cells and is responsible for the production of prostaglandins that maintain homeostasis; however, COX-2 is upregulated in inflammatory cells in response to an inflammatory stimulus and is responsible for the production of prostaglandins at the site of inflammation (Crofford 1997; Masferrer and Seibert 1994; Mitchell *et al*., 1993; Seibert and Masferrer 1994). Therefore, there is an increasing interest in the usefulness of COX-2 inhibitors.

Currently prescribed treatments for inflammation include steroidal and non-steroidal anti-inflammatory drugs, but these conventional drugs have not been successful against chronic inflammatory disorders such as rheumatoid arthritis and atopic dermatitis. The lack of knowledge of the critical etiology and exacerbating mechanisms of inflammatory disorders has delayed the development of new anti-inflammatory drugs (Kim*et al*., 2004). Therefore, the use of traditional medicinal plants or their crude extracts is increasingly becoming an attractive complementary or alternative medicine approach for the treatment of various inflammatory disorders (Lee *et al*., 2001). Focusing on the anti-inflammatory properties of certain plants, we found that *I. dentata* (IXD, Asteraceae) potently suppressed UVB damage. The whole plant of IXD, a typical Oriental herb, has been used for the treatment of indigestion, pneumonia, hepatitis, contusion, and tumor (S. Kim 1995; M. Kim and M. Lee 1988). It has also been usedas a folk therapy in Korea for the treatment of inflammatory diseases. IXD is known to have aliphatics, triterpenoids, and sesquiterpene glycosides in its composition (Arai *et al*., 1983). In previous studies, IXD was shown to inhibit nitric oxide and tumor necrosis factor-α activity (Chung *et al*., 2002; Jung *et al*., n.d.). However, none of these has provided any information about the effect of IXD on skin inflammation.

Therefore, in the present study, we studied the potential anti-inammatory action of IXD ethyl acetate fraction (IXDE). We examined its influence on UVB-induced pro-inflammatory cytokine production in HaCaT cells by evaluating cells that were stimulated with UVB in the presence or absence of IXDE.

Experimental

Plant Material and Extract Preparation – IXD was purchased from the company of Jeong Dong Myong Sseumbagwi. According to the scheme, dried whole IXD plants (100 g) were extracted with 70% EtOH 3 times for 2 h under heating mantle reflux. The resultant extract was condensed to 1 L in a rotary vacuum evaporator (N-1000S, EYELA, Tokyo, Japan). Finally, the 70% extract (42.7 g) was suspended in water and then partitioned successively with organic solvents of different polarities to obtain n-hexane (6.1 g), choloroform (5.1 g), ethyl acetate (3.6 g), n-butanol (7.3 g), and aqueous (9.1 g) fractions (Fig. 1).

Reagents – RPMI 1640, penicillin, and streptomycin were obtained from Hyclone (Logan, UT, USA). Bovine serum albumin and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) were purchased from Sigma (St. Louis, MO, USA). Antibodies against p38, phosphorylated p38, JNK, phosphorylated JNK, and β-actin, and peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibodies against human IL-6 and IL-8 and biotinylated antibodies against human IL-6 and IL-8 were purchased from BD Biosciences (San Jose, CA, USA). The RNeasy Mini kit and QuantiTect Reverse Transcription kit were purchased from QIAGEN (Hilden, Germany). Finally, IL-6, IL-8, COX-2, and β-actin oligonucleotide primers were purchased from Bioneer Corp. (Daejung, Republic of Korea).

Cell Culture – HaCaT cells were grown in RPMI 1640 medium containing 5% fetal bovine serum and 100 U/ml of penicillin/streptomycin sulfate. The cells were incubated in a humidified 5% CO_2 atmosphere at 37 °C.

UVB Source – UVB irradiation was delivered by a closely spaced array of 5 sunlamps (G9T5E lamps, Sankyo Denki Co., Hiratsuka, Japan). The distance between the sunlamps and the surface of the cell cultures was fixed at 7.5 cm, and the distance between the sunlamps and the surface of the cage was fixed at 30 cm. The energy output of the UVB (290 - 320 nm) lamps was measured with a

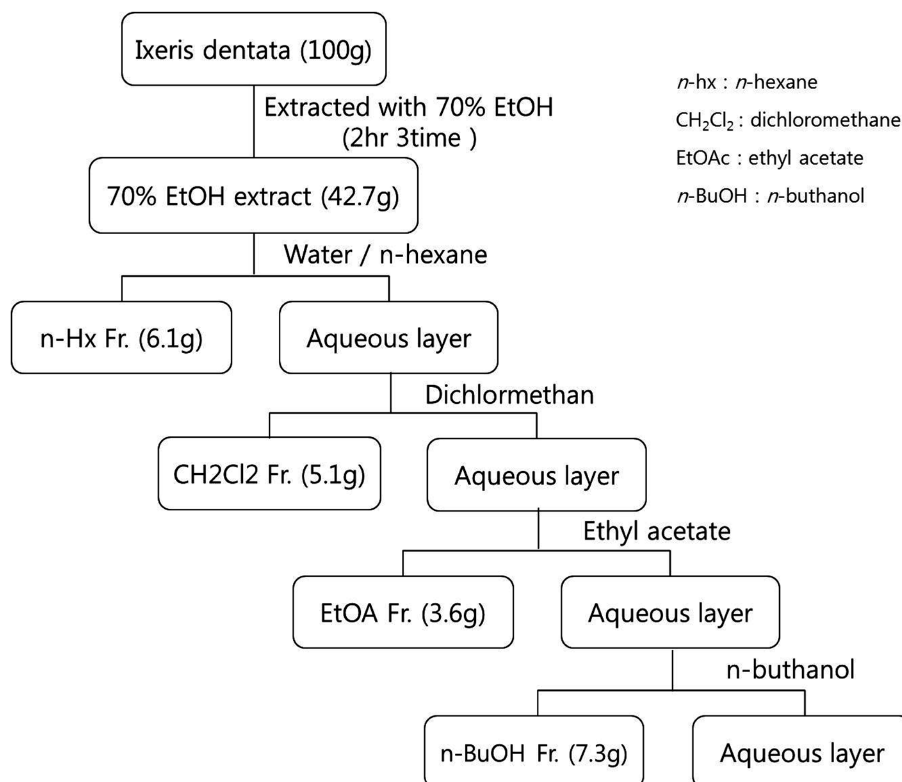

Fig. 1. Preparation of *Ixeris dentata* fractions.

UV radiometer (VLX-3W, VilberLourmat, Marne-la-Vallee, France).

Cell Viability Assay – Cell viability was determined by the MTS assay. HaCaT cells were plated at a density of 3×10^4 cells/well in 96-well plates (Nunc, Copenhagen, Denmark). Each experiment included a non-treated group as control. To determine the non-toxic concentration for cells, IXDE (10, 50, 80, 100, and 200 µg/mL) was then added to each well. The plates were then incubated for 24 h at 37 °C under 5% CO_2. The MTS solution (5 mg/mL) was added to each well and the cells were cultured for another 2 h, after which the optical density was read at 490 nm. Cytotoxicity was then calculated using the formula:1-(mean absorbance value of treated cells/mean absorbance value of untreated cells).

Enzyme-Linked ImmunosorbentAssay – Cells were seeded at a density of 3×10^4/well in 48-well tissue culture plates and pretreated with various concentrations of IXDE (50, 80, and 100 µg/mL) for 24 h before UVB (100 mJ/cm^2) stimulation. Enzyme-linked immunosorbent assay (ELISA) plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) were coated overnight at 4 °C with anti-human IL-6 and IL-8 antibodies diluted in coating buffer (0.1 M carbonate, pH 9.5) and then washed 3times with phosphate-buffered saline (PBS) containing 0.05% Tween 20. Nonspecific protein binding sites were blocked with assay diluent (PBS containing 10% fetal bovine serum, pH 7.0) for at least 1 h. Immediately, each sampleortheIL-6 or IL-8 standard was added to the wells. After incubation for 2 h, a working detector was added and incubated for 1 h. Accordingly, the substrate solution (tetramethylbenzidine) was added to the wells and incubated for 30 minutes in the dark before the reaction was stopped using the stop solution (2 N H_3PO_4). The absorbance was read at 450 nm. All subsequent steps took place at room temperature, and all standards and samples were assayed in duplicate.

Western Blot Analysis – Protein expression was assessed by western blot analysis according to standard procedures. The HaCaT cells were cultured in 60-mm-diameter culture dishes (4×10^6/well) and pretreated with various concentrations of IXDE (50, 80, and 100 µg/mL). After 30 min, 2 h, or 24 h, the cells were UVB-irradiated (100 mJ/cm^2), and then incubated at 37 °C. After incubation, the cells were washed twice in ice-cold PBS (pH 7.4). The cell pellets were resuspended in lysis buffer on ice for 20 minutes, and the cell debris was removed by centrifugation. The protein concentrations were determined

using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Equal amounts of protein (20 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene membrane (Millipore, Bedford, MA, USA).The membrane was blocked with 5% nonfat milk in Tris-buffered saline with Tween 20 buffer (150 mM NaCl, 20 mM Tris-HCl, and 0.05% Tween 20, pH 7.4). After blocking, the membrane was incubated with primary antibodies for 18 h. The membrane was then washed with Tris-buffered saline with Tween 20 and incubated with anti-mouse or anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibodies. Immunoreactivity was detected using enhanced chemiluminescence (Amersham, Milan, Italy).

RNA Extraction and Reverse-Transcription Polymerase Chain Reaction (RT-PCR) – HaCaT cells were cultured in 6-well tissue culture plates (8×10^5/well) and pretreated with various concentrations of IXDE (50, 80, and 100 μg/mL). After 30 minutes, the cells were irradiated with UVB (100 mJ/cm^2), and then incubated at 37 °C. After incubation, the cells were washed twice in ice-cold PBS (pH 7.4). Total cellular RNA was isolated using the RNeasy Mini kit (Qiagen) and 1 μg of total RNA was reverse-transcribed using the QuantiTect Reverse Transcription kit (Qiagen), according to the manufacturer's instructions. The total RNA (2 μg) was converted to cDNA by treating it with 200 units of reverse transcriptase and 500 ng of oligo (dT) primer in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, and 1 mM deoxynucleotide triphosphates at 42 °C for 1 h. The reaction was stopped by heating at 70 °C for 15 minutes, and the cDNA mixture (3 μL) was used for enzymatic amplification. PCR was performed in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, 2.5 units of Taq DNA polymerase, and 0.1 μM each of the IL-6, IL-8, COX-2, or β-actin primers, respectively. The conditions for amplification were as follows: an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing for 30 sat either 58 °C (for IL-6 and COX-2 primers) or 55 °C (for IL-8 primers), and extension at 72 °C for 30 s. The final extension was performed at 72 °C for 7 minutes. PCR products were electrophoretically separated on a 2% agarose gel and stained with ethidium bromide.

Statistical Analysis – Statistical analysis was performed using one-way analysis of variance (ANOVA) or Student's t-test for single comparisons. All data are presented as mean ± standard error (SE), and the number of individual experiments conducted is mentioned in each figure legend.

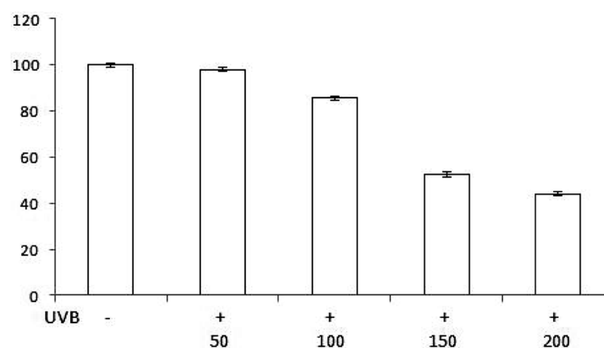

Fig. 2. Viability of HaCaT cells under various conditions. HaCaT cells were used for the MTS assay at 24 h after 50 mJ/cm^2, 100 mJ/cm^2, or 150 mJ/cm^2 UVB irradiation and comparison of the viability of irradiated cells with that of the non-irradiated control. Data are mean ± SD values from triplicate experiments.

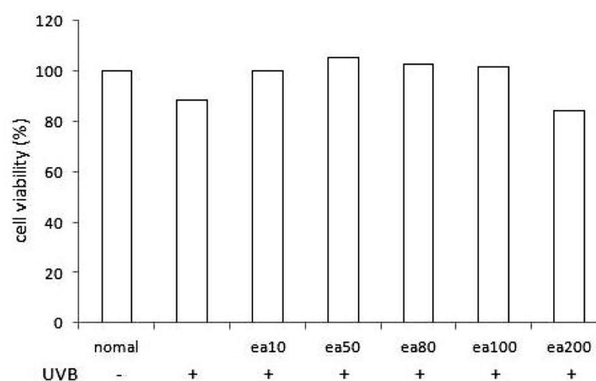

Fig. 3. Effects of IXDE on cell viability in HaCaT cells. Cell viability was evaluated using the MTS assay. Data represent the mean (SE) of duplicate measurements from 3 separate experiments.

Results

Cell viability of UVB-irradiated HaCaT cells – The effect of IXDE on cell viability following UVB irradiation was tested in HaCaT cells. Cell viability was evaluated using an MTS assay under various conditions (Fig. 2). When cultures were incubated after UVB irradiation, UVB-induced toxicity increased compared to non-irradiated cells. Cell viability declined in a UVB irradiation dose-dependent manner and was sharply reduced at 24 h after UVB irradiation of 150 mJ/cm^2. Accordingly, we selected an exposure dose of 100 mJ/cm^2 to study cellular toxicity in HaCaT cells treated with IXDE 24 h after UVB irradiation (Fig. 3).

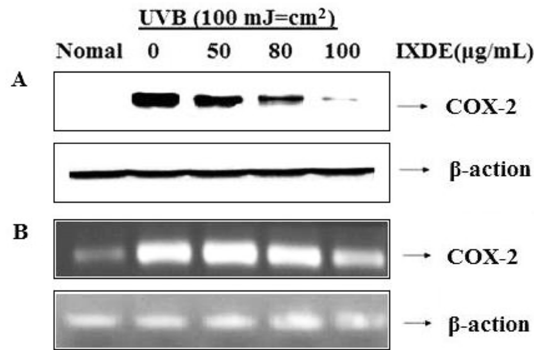

Fig. 4. Effect of IXDE on UVB-induced COX-2 protein and mRNA expression in HaCaT cells. HaCaT cells were pretreated with the indicated concentrations of IXDE for 24 h before being irradiated with UVB (100 mJ/cm^2) for 24 h. Equal amounts of protein (20 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with COX-2 antibodies. Equal protein loading was verified using β-actin. (A) COX-2 mRNAs were assessed by RT-PCR in HaCaT cells. Cells were pretreated with the indicated concentrations of IXDE for 24 h before being incubated with UVB (100 mJ/cm^2) for 24 h. β-actin mRNA was assayed in parallel to confirm the equivalency of the cDNA preparations. (B) The experiment was repeated 3 times, and similar results were obtained. **$P < 0.005$, when compared to the UVB treated group. Significant differences between treated groups were determined using the Student's t-test. Values shown are the mean ± S.E. of duplicate determinations from 3 separate experiments.

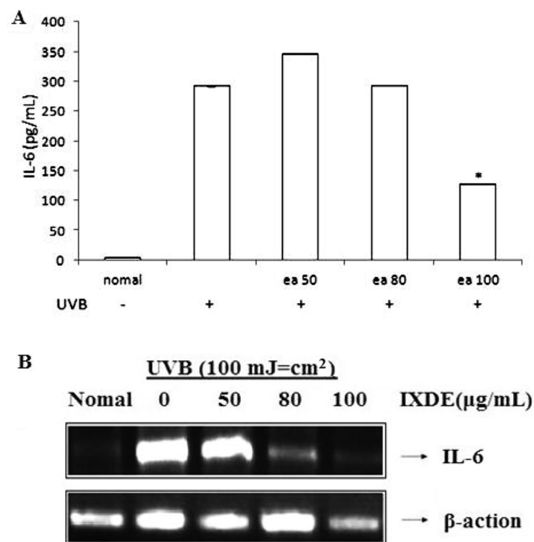

Fig. 5. Effect of IXDE on UVB-induced IL-6 production. HaCaT cells were pretreated with the indicated concentrations of IXDE for 24 h before being irradiated with UVB (100 mJ/cm^2) for 24 h. The production of IL-6 was measured by ELISA. Cells were pretreated with the indicated concentrations of IXDE for 24 h before being irradiated with UVB (100 mJ/cm^2) for 24 h. (A) Effect of IXDE on UVB-induced IL-6 mRNA expression. IL-6 mRNA was assessed by RT-PCR in HaCaT cells. Cells were pretreated with the indicated concentrations of IXDE for 24 h before being irradiated with UVB (100 mJ/cm^2) for 2 h. β-actin mRNA was assayed in parallel to confirm the equivalency of the cDNA preparations. (B) Data are mean ± SE values of duplicate determinations from 3 separate experiments. *$P < 0.5$; **$P < 0.005$.

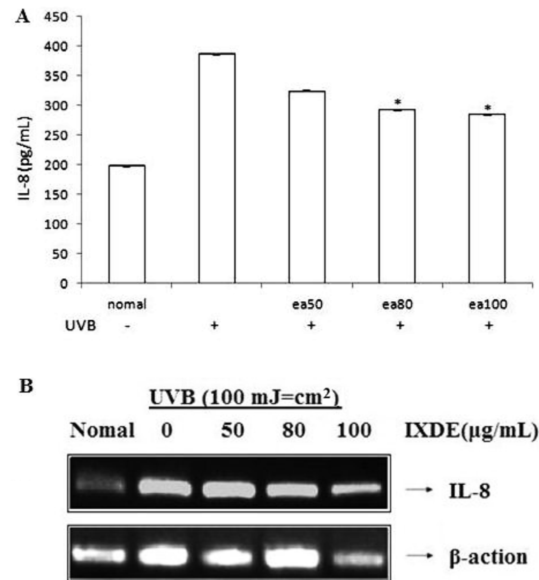

Fig. 6. Effect of IXDE on UVB-induced IL-8 production. HaCaT cells were pretreated with the indicated concentrations of IXDE for 24 h before being irradiated with UVB (100 mJ/cm^2) for 24 h. The production of IL-8 was measured by ELISA. Cells were pretreated with the indicated concentrations of IXDE for 24 h before being incubated with UVB (100 mJ/cm^2) for 24 h. (A) Effect of IXDE on UVB-induced IL-8 mRNA expression. IL-8 mRNA was assessed by RT-PCR in HaCaT cells. Cells were pretreated with the indicated concentrations of IXDE for 24 h before being irradiated with UVB (100 mJ/cm^2) for 2 h. β-actin mRNA was assayed in parallel to confirm the equivalency of the cDNA preparations. (B) Data are the mean ± SE values of duplicate determinations from 3 separate experiments. *$P < 0.05$. **$P < 0.005$

Effect of IXDE on IL-6 and IL-8 production in UVB-irradiated cells – Since IXDE inhibited the production of pro-inflammatory mediators in HaCaT cells, we further investigated its effects on UVB-induced IL-6 and IL-8 production, using ELISA and RT-PCR. We found that IXDE inhibited UVB-stimulated IL-6 expression at both the protein and mRNA levels in a concentration-dependent manner (Fig. 5 and 6 A and B).

Effect of IXDE on UVB-induced COX-2 mRNA expression – We next examined the effects of IXDE on COX-2 expression in UVB-induced HaCaT cells. The expression of COX-2 protein and COX-2 mRNA were measured in HaCaT cells exposed to UVB (100 mJ/cm^2) for 24 h. IXDE effectively suppressed the induction of COX-2 by UVB (Fig. 3B). UVB (100 mJ/cm^2) also increased COX-2 mRNA expression, which was inhibited in the presence of IXDE (Fig. 4, A and B). Hence, IXDE suppressed the expression of genes that are implicated in the pathogenesis of inflammatory responses.

Effects of IXDEon the phosphorylation of MAPKs in UVB-induced HaCaT cells – MAPKs are essential for

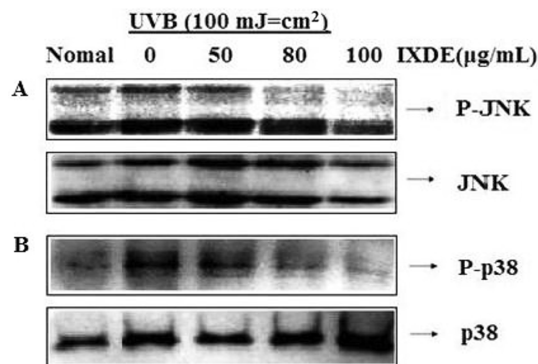

Fig. 7. Effect of IXDE on the phosphorylation (P-) of MAPKs in UVB-stimulated HaCaT cells. HaCaT cells were treated with the indicated concentrations of IXDE for 24 h before being irradiated with UVB (100 mJ/cm^2) for 30 min (A) and 1 h (B). Whole-cell lysates were analyzed by western blot analysis. The experiment was repeated 3 times, and similar results were obtained.

UVB-induced inflammation in HaCaT cells. Therefore, we evaluated the effects of IXDE on the activation of MAPKs in UVB-stimulated HaCaT cells. As shown in Fig. 7, panels A and B, IXDE markedly inhibited the phosphorylation of JNK 1/2 and p38 MAPK; these results indicate that MAPK phosphorylation was inhibited by IXDE pretreatment.

Discussion

Ultraviolet B irradiation induces skin damage and inflammation through the secretion of various cytokines, which are immune regulators produced by cells. To prevent the initiation of skin inflammation, keratinocytes that have been irreversibly damaged by UVB must be removed through the apoptotic mechanism. IXD is a perennial medicinal herb indigenous to Korea. We examined the influence of IXD on UVB-induced pro-inflammatory cytokine production in HaCaT cells by evaluating cells that were stimulated with UVB in the presence or absence of IXD. In the present study, pro-inflammatory cytokine production was measured by ELISA, RT-PCR, and the activation of MAPKs as determined by western blot analysis. IXD is known to contain aliphatics, triterpenoids, and sesquiterpene glycosides in its composition (Arai *et al*., 1983). In previous studies, IXD was found to inhibit the activities of nitric oxide and tumor necrosis factor-α (Chung *et al*., 2002; Jung *et al*., 2007). However, none of these has provided any information about the effect on skin inflammation.

In the present study, we evaluated the potential anti-inflammatory action of IXDE in HaCaT cells. Specifically, we investigated whether it inhibits the UVB-induced production of IL-6 and IL-8 by inhibiting the expression of MAPK and COX-2 at the protein and mRNA levels. We found that the inhibitory effects of IXDE on the production of inflammatory mediators were accompanied by concentration-dependent decreases in the protein and mRNA expression levels of IL-6, IL-8, and COX-2. These data demonstrate that IL-6, IL-8, and COX-2 expression in HaCaT cells is suppressed by IXDE.

MAPKs (ERK 1/2, JNK 1/2, and p38 MAPK) play critical roles in cell growth regulation and differentiation and in the control of cellular responses to cytokines and stressors (Johnson and Lapadat 2002).We found that IXDE inhibited the UVB-induced phosphorylation of JNK 1/2 and p38in HaCaT cells.

In conclusion, we have shown that IXDE exerts inhibitory effects on UVB-induced IL-6 and IL-8 production in HaCaT cells. Furthermore, these effects are mediated by the inhibition of COX-2 expression and JNK 1/2 and p38 phosphorylation. In practice, the whole plant of IXD, a typical Oriental herb, has been used for treatment of indigestion, pneumonia, hepatitis, contusion, and tumor (Kim 1995; Kim and Lee 1988). It has also been used for the treatment of inflammatory diseases as a folk therapy in Korea. Therefore, this represents a potent anti-inflammatory effect of IXDE through blockades on inflammatory mediators. Our data suggest that IXDE represents a new source of potential drugs for the treatment of inflammatory diseases. Further research is required in order to purify and identify the structure of the anti-inflammatory component of IXDE and to determine the mechanisms by which it exerts its anti-inflammatory action.

Acknowledgement

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