Protective effect of ixerisoside A against UVB-induced pro-inflammatory cytokine production in human 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), one of the harmful radiations for skin, is widely known to induce abnormally increased cytokine release from keratinocytes leading to inflammatory skin disorders. IL-6 and IL-8 induce an acute-phase response and stimulate leukocyte infiltration in the skin. Previous studies have shown that chronic exposure to UVB radiation increases cyclooxygenase-2 (COX-2) expression through various cell signaling pathways, resulting in skin cancer. Recent studies have shown that the activation of extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38 MAPK is strongly correlated with acute inflammation and development of skin cancer caused by an increased expression of COX-2. Ixerisoside A (IXA) is an active constituent of *Ixeris dentata* of the Compositae (Asteraceae) family. The effect of IXA on skin inflammation has yet to be elucidated. To determine the anti-inflammatory effects of IXA, we examined its effect on UVB-induced pro-inflammatory cytokine production in human keratinocytes (HaCaT cells) by observing these cells in the presence or absence of IXA. In this study, pro-inflammatory cytokine production was determined by enzyme-linked immunosorbent assay (ELISA), reverse transcription-polymerase chain reaction (RT-PCR), and western blot analysis to evaluate the activation of mitogen-activated protein kinases (MAPKs). IXA inhibited UVB-induced production of the pro-inflammatory cytokines IL-6 and IL-8 in a dose-dependent manner. Moreover, IXA inhibited the expression of COX-2, ERK, JNK, and p38 MAPKs, indicating that the secretion of the pro-inflammatory cytokines IL-6 and IL-8, and COX-2 expression was inhibited by blocking MAPK phosphorylation. These results indicated that IXA potentially protects against UVB-induced skin inflammation.

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Introduction

Keratinocytes are the main cells found in the epidermal layer of the skin and are exposed to a variety of stress stimuli such as ultraviolet (UV) radiation (1,2). Solar UV radiation is divided into three main wavelength ranges: UVC (λ=100-290 nm), ultraviolet B (UVB) (λ=290-320 nm), and UVA (λ=320-400 nm) (3,4). Although UVC is completely absorbed by the ozone layer, UVA and UVB both reach the Earth's surface in sufficient amounts to have damaging effects on skin (5). As an environmental factor, UVB radiation has numerous effects on human health. UVB exposure is necessary to produce vitamin D in the skin. As vitamin D deficiency causes immune dysfunction, nervous system, bone growth, cell proliferation, insulin secretion, and blood pressure, regular and minimum exposure of skin to UVB is required for maintaining optimal health (6). However, UVB in particular has a wide spectrum of biological effects on the skin, and its acute exposure causes a variety of adverse skin reactions such as erythema, edema, sunburn, hyperplasia, inflammation, and immunosuppression (7). UV-irradiated cells in inflammatory infiltration and various non-epidermal skin cells also produce cytokines, many of which may act on keratinocytes. The regulation and function of cytokines in keratinocytes have been extensively studied and reviewed. These pro-inflammatory cytokines are considered to be closely associated with the progression of photodamage. Among keratinocyte-derived cytokines, the pro-inflammatory cytokines IL-1β, IL-6, TNF-α, and IL-8 have been well characterized (8,9). IL-6 and

IL-8 induce an acute-phase response and stimulate leukocyte infiltration in the skin (10).

Cyclooxygenase-2 (COX-2) is the rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandins. COX-2 expression is induced by inflammatory cues in various tissues such as the epidermis (11,12). Previous findings have shown that chronic exposure to UVB radiation increases COX-2 expression through various cell signaling pathways, resulting in the induction of skin cancer (13,14). Mitogen-activated protein kinases (MAPKs) are a family of proline-directed Ser/Thr kinases comprising extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38 MAPK. The activation of ERK, JNK, and p38 MAPK was found to be strongly correlated with acute inflammation and development of skin cancer through an increased expression of COX-2 (15-17).

Ixeris dentata family Compositae (Asteraceae), a typical medicinal herb, has been used for the treatment of indigestion, pneumonia, hepatitis, contusions, and tumors (18). Ixerisoside A (IXA) is an active constituent isolated from the extract of *Ixeris dentata* and contains aliphatic compounds, triterpenoids, and sesquiterpene glycosides (19).

Pharmacological studies on *Ixeris dentata* showed that water or organic extracts of whole herbal medicine decreased lipid concentrations and acted as an antioxidant, antiallergic, monamine oxidase, anti-inflammatory, antimutagenic, and anticancer activity (20-24). In particular, previous findings showed that *Ixeris dentata* exerted a protective effect against UVB-induced skin inflammation (25,26). Therefore, in this study, we examined the potential anti-inflammatory action of IXA. We examined its effect on UVB-induced pro-inflammatory cytokine production in human keratinocytes (HaCaT cells) by evaluating UVB-stimulated cells in the presence or absence of IXA.

Materials and methods

Plant material. Whole plants of *Ixeris dentata* family Compositae (Asteraceae) were collected in May 2006 from the herbarium at the Korea Research Institute of Chemical Technology (KRICT) and were authenticated by Dr Young Sup Kim. A voucher specimen (KR0472) was deposited into the herbarium at KRICT (27).

Extraction and isolation. The air-dried whole plants (6 kg) of *Ixeris dentata* were soaked in methanol (MeOH) (2x40 l) at room temperature for 7 days. The MeOH extract was filtered and evaporated to dryness under reduced pressure. The concentrated extract (840 g) was suspended in 20 l of water and then extracted successively with an equal volume of dichloromethane (MC), ethyl acetate (EtOAc), and n-butanol (n-BuOH) to afford 160, 15 and 60 g of the compound in MC, EtOAc, and n-BuOH fractions, respectively (27). The suggested chemical structure of IXA is shown in Fig. 1.

Reagents. RPMI-1640, penicillin, and streptomycin were obtained from HyClone Laboratories, Inc. (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-Aldrich (St. Louis, MO, USA). Antibodies for ERK, phosphorylated ERK, JNK, phosphorylated JNK, p38, phosphorylated p38, and β-actin, and peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies for human IL-6 and IL-8 and biotinylated antibodies for 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). IL-6, IL-8, COX-2, and β-actin oligonucleotide primers were purchased from Bioneer Corp. (Daejeon, Korea).

Cell culture. HaCaT cells were grown in RPMI-1640 medium containing 5% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin sulfate. The cells were incubated in 5% humidified CO_2 atmosphere at 37˚C. To stimulate the cells, the medium was replaced with fresh RPMI-1640 medium and exposed to UVB in the presence or absence of IXA for the indicated time points.

UVB source. UVB irradiation was delivered using a closely spaced array of five sunlamps (G9T5E lamps; Sankyo Denki Co., Ltd., 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 using a UV radiometer (UVX; UVP Inc., Upland, CA, USA).

MTS assay for cell viability. Cell viability was determined by the MTS assay. HaCaT cells were plated at a density of $3x10^4$ cells/well in 96-well plates (Thermo Scientific Nunc®, Nunc AS, Copenhagen, Denmark). Each experiment had a non-treated group as the control. To determine the non-toxic concentration for the cells, IXA (2.5, 5, 10, and 20 μM) was added to each well. The plates were incubated for 24 h at 37˚C under 5% CO_2. MTS solution (5 mg/ml) was added to each well, and the cells were cultured for another 2 h, followed by measuring their optical density at 490 nm. The cytotoxicity was calculated using the formula: 1 - (mean absorbance value of treated cells/mean absorbance value of untreated cells).

Enzyme-linked immunosorbent assay. Cells were seeded at a density of $3x10^4$ cells/well in 48-well tissue culture plates and pre-treated with two concentrations of IXA (5 and 10 μM) for 24 h prior to UVB (100 mJ/cm^2) stimulation. The culture supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA) for IL-6 and IL-8 levels. To measure the cytokines, a modified ELISA method was used. First, a sandwich ELISA for IL-6 and IL-8 was conducted in duplicate in 96-well immuno plates (Nunc 439454; Thermo Scientific Nunc®). The supernatant was decanted into a new microcentrifuge tube, and the cytokines were quantified by ELISA. ELISA plates were coated overnight at 4˚C with anti-human IL-6 and IL-8 monoclonal antibodies diluted in coating buffer (0.1 M carbonate, pH 9.5), and then washed four times with phosphate-buffered saline (PBS) containing 0.05% Tween-20. The non-specific protein binding sites were blocked with assay diluent (PBS containing 10% FBS at pH 7.0) for at least 1 h. After washing the plates again, the test sample or recombinant

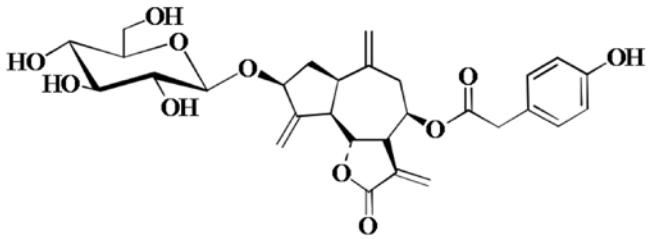

Figure 1. The chemical structure of ixerisoside A (IXA).

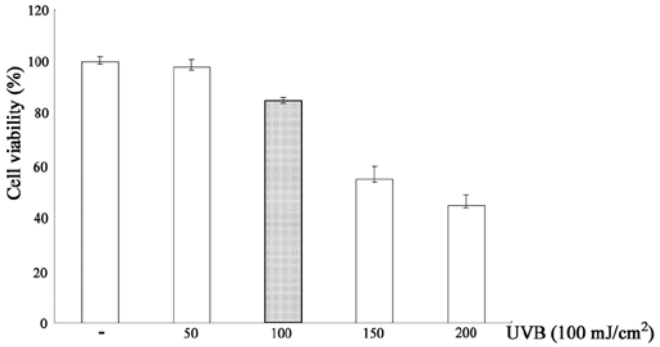

Figure 2. Viability of human keratinocytes (HaCaT cells) under various conditions. HaCaT cells were used for the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay at 24 h after 50, 100, 150 or 200 mJ/cm^2 ultraviolet B (UVB) irradiation and a comparison was made of the viability of irradiated cells with that of the non-irradiated control. Data are the means ± standard error (SE) values from triplicate experiments.

IL-6 and IL-8 standards was added. After incubation for 2 h, a working detector (biotinylated anti-human IL-6 and IL-8 monoclonal antibodies and streptavidin-horseradish peroxidase reagent) was added, and the mixture was incubated for 1 h. Subsequently, the substrate solution (tetramethylbenzidine) was added to the wells and incubated for 30 min in the dark before the reaction was quenched by adding 1 M H_3PO_4. The absorbance was read at 450 nm using an ELISA reader (Infinite M200; Tecan, Männedorf, Switzerland). Subsequent steps were conducted at room temperature, and the standards and samples were assayed in duplicate.

Western blot analysis. Protein expression was assessed by western blot analysis according to standard procedures. HaCaT cells were cultured in 60-mm-diameter culture dishes (4x10^6 cells/well) and pre-treated with two concentrations of IXA (5 and 10 μM). After 30 min, 2 or 24 h, the cells were UVB-irradiated (100 mJ/cm^2) and then incubated at 37˚C. Following the incubation, the cells were washed twice in PBS (pH 7.4) and were resuspended in lysis buffer on ice for 20 min. The cell debris was removed by centrifugation (10,000 x g, 10 min, and 4˚C). 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 sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene membrane (Millipore, Bedford, MA, USA). The membrane was blocked using 5% non-fat 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. Antibodies against ERK, phosphorylated ERK, JNK, phosphorylated JNK, p38, phosphorylated p38 and β-actin, and peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. The membrane was then washed with Tris-buffered saline containing Tween-20 and incubated with anti-mouse or anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibodies. The proteins were then supplemented with ECL Prime Western Blotting Detection Reagents and an ImageQuant LAS 4000 mini biomolecular imager (both from GE Healthcare, Cleveland, OH, USA) was used to evaluate the bands.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total cellular RNA was isolated using an easy-BLUE™ RNA extraction kit (Intron Biotechnology, Inc., Seongnam, Republic of Korea) according to the manufacturer's instructions. The total RNA (2 μg) was then 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 quenched by heating the samples at 70˚C for 15 min, followed by enzymatic amplification of the cDNA mixture (3 μl). PCR was conducted in a reaction mixture containing 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 IL-6, IL-8, COX-2, and GAPDH primers. PCR of GAPDH was conducted by subjecting the reaction mixtures to an initial denaturation of 92˚C for 5 min, followed by 30 cycles of 92˚C for 1 min, annealing at 50˚C for 1 min, and extension at 72˚C for 2 min, with a final extension at 72˚C for 5 min. PCR of IL-6 and IL-8 samples was conducted by heating the reaction mixtures to 95˚C for 2 min, followed by 35 cycles at 95˚C for 45 sec, 60˚C for 45 sec, and 72˚C for 90 sec, with a final extension at 72˚C for 10 min. PCR of COX-2 samples was conducted by heating the reaction mixtures to 94˚C for 5 min, followed by 30 cycles at 94˚C for 1 min, 55˚C for 30 sec, and 72˚C for 1 min, with a final extension at 72˚C for 7 min. The PCR products were then electrophoresed on 1% agarose gel and stained with ethidium bromide. The primer sequences are listed in Table I.

Statistical analysis. Statistical analysis was performed using one-way analysis of variance (ANOVA) or the Student's t-test for single comparisons. Data are presented as the means ± standard error (SE), and the number of individual experiments conducted is mentioned in each figure legend. $P<0.05$ and $P<0.005$ were considered to indicate statistically significant differences.

Results

Cell viability of UVB-irradiated HaCaT cells. The effect of IXA on cell viability following UVB irradiation was assessed on HaCaT cells. Cell viability was evaluated using the MTS

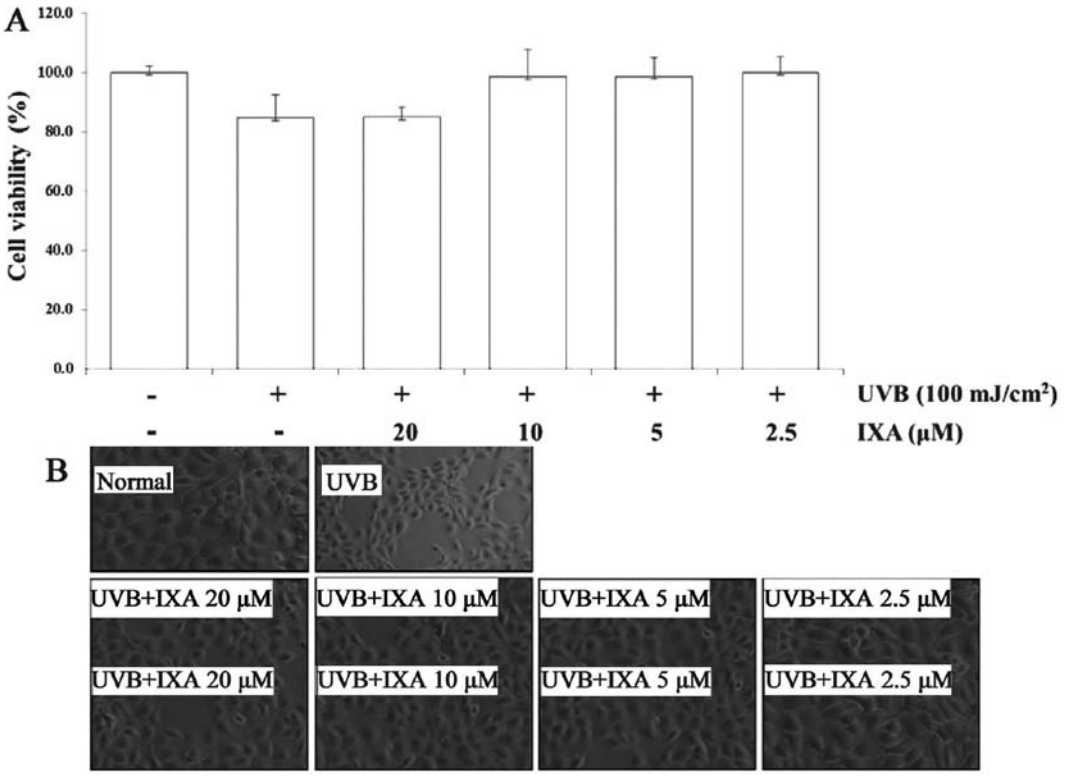

Figure 3. Effect of ixerisoside A (IXA) on cell viability and morphological alterations in human keratinocytes (HaCaT cells). (A) Cell viability was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. Data are the means ± standard error (SE) of duplicate determinations from three separate experiments. (B) For morphological studies, the cells were treated with IXA (2.5, 5, 10 and 20 μM) for 24 h and then stimulated with ultraviolet B (UVB) (100 mJ/cm^2) for 24 h.

Table I. Sequences of oligonucleotide primers designed for PCR.

cDNA	Sequences
IL-6	F, 5'-ATGAACTCCTTCTCCACAAGCGC-3'
	R, 5'-GAAGAGCCCTCAGGCTGGACTG-3'
IL-8	F, 5'-ATGACTTCCAAGCTGGCCGTGGCT-3'
	R, 5'-TCTCAGCCCTCTTCAAAAACTTCTC-3'
COX-2	F,5'-TTCAAATGAGATTGTGGGAAAATTGCT-3'
	R, 5'-AGATCATCTCTGCCTGAGTATCTT-3'
GAPDH	F, 5'-CTGGCACCCAGCACAATGAAG-3'
	R, 5'-ACCGACTGCTGTCACCTTCA-3'

F, forward; R, reverse; COX-2, cyclooxygenase-2.

assay (Fig. 2). When the cultures were incubated after UVB irradiation, UVB-induced toxicity increased compared to that in non-irradiated cells. Cell viability decreased depending on the dose of UVB irradiation and was markedly reduced at 24 h after UVB irradiation of 150 mJ/cm^2. Subsequently, an exposure dose of 100 mJ/cm^2 was selected to study cell toxicity in HaCaT cells treated with IXA 24 h after UVB irradiation (Fig. 3).

Effects of IXA on the production of pro-inflammatory cytokines. Initially, the cytotoxicity of IXA on HaCaT cells was examined using the MTS assay. The half maximal inhibitory concentration (IC_{50}) value of IXA was 50 μM (data not shown), and IXA did not show any cytotoxic effects up to 10 μM (Fig. 3). To evaluate the effect of IXA on the production of pro-inflammatory cytokines, the cells were pre-treated with IXA (5 and 10 μM) prior to stimulation by UVB (100 mJ/cm^2) for 24 h and analyzed by ELISA. As shown in Fig. 4, the levels of IL-6 and IL-8 were considerably increased in HaCaT cells after stimulation with UVB (100 mJ/cm^2). Pre-treatment of cells with IXA (5 or 10 μM) inhibited these increments in a concentration-dependent and statistically significant manner.

Effects of IXA on pro-inflammatory cytokine gene expression. The pro-inflammatory cytokine gene expression was then analyzed using RT-PCR. Enhanced IL-6 and IL-8 mRNA expression induced by UVB (100 mJ/cm^2) was inhibited by pre-treatment of the cells with IXA (Fig. 5). In particular, the pre-treatment with IXA at a concentration of 10 μM inhibited the UVB-induced gene expression of IL-6 and IL-8.

Effects of IXA on COX-2 protein and mRNA expression. The effects of IXA on COX-2 expression were examined in UVB-irradiated HaCaT cells. The expression levels of COX-2 protein and mRNA were measured in HaCaT cells exposed to UVB (100 mJ/cm^2) for 24 h. IXA effectively suppressed UVB-induced COX-2 expression. UVB (100 mJ/cm^2) also increased COX-2 mRNA expression, which was inhibited in the presence of IXA (Fig. 6). Thus, IXA suppressed the

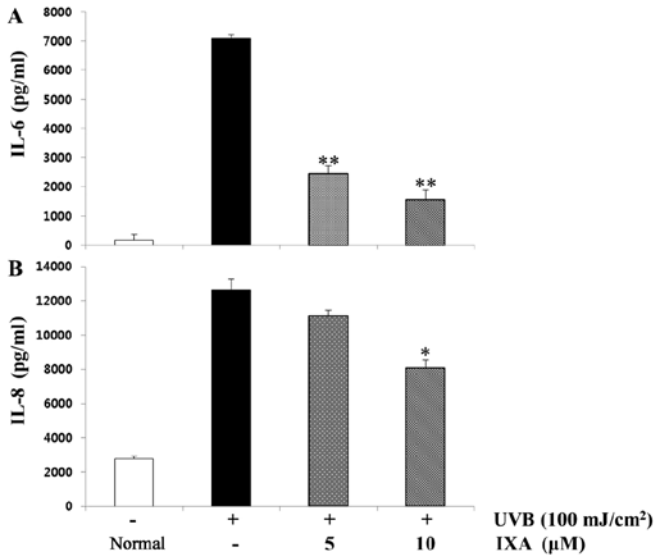

Figure 4. Effect of ixerisoside A (IXA) on ultraviolet B (UVB)-induced (A) IL-6 and (B) IL-8 production. Human keratinocytes (HaCaT cells) were pre-treated with the indicated concentrations of IXA for 24 h prior to being irradiated with UVB (100 mJ/cm^2) for 24 h. The production of IL-6 and IL-8 was measured by ELISA. The cells were pre-treated with the indicated concentrations of IXA for 24 h before being irradiated with UVB (100 mJ/cm^2) for 24 h. Data are the means ± standard error (SE) values of duplicate determinations from three separate experiments. *P<0.05, **P<0.005.

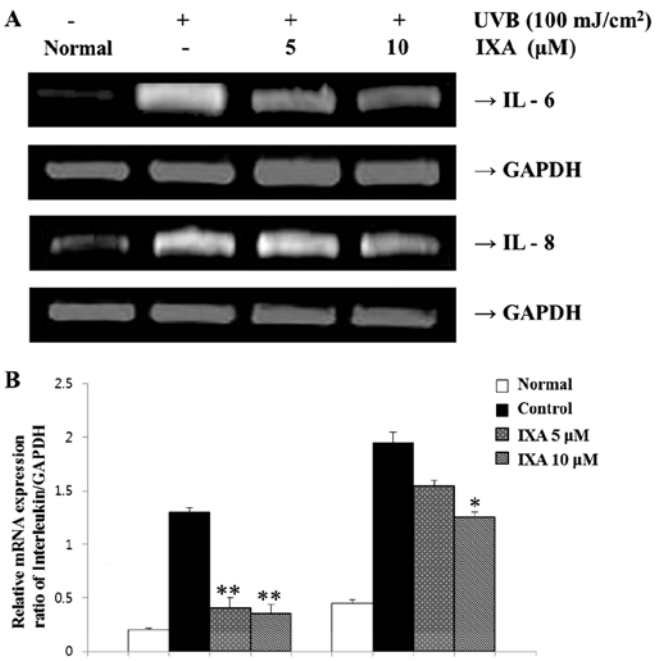

Figure 5. Effect of ixerisoside A (IXA) on ultraviolet B (UVB)-induced IL-6 and IL-8 mRNA expression. (A) Human keratinocytes (HaCaT cells) were pre-treated with the indicated concentrations of IXA for 24 h before being irradiated with UVB (100 mJ/cm^2) for 24 h. IL-6 mRNA was assessed by reverse transcription-polymerase chain reaction (RT-PCR) in HaCaT cells. (B) Cells were pre-treated with the indicated concentrations of IXA 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. Data are the means ± standard error (SE) values of duplicate determinations from three separate experiments. *P<0.05, **P<0.005.

expression of genes that were involved in the pathogenesis of inflammatory responses.

Effects of IXA on the activation of MAPKs. Inhibition of the ERK, JNK, and p38 MAPK pathways was recently found to attenuate pro-inflammatory cytokine secretion. The effect of IXA on UVB-induced MAPK phosphorylation in HaCaT cells was examined by incubating the cells with IXA 24 h prior to stimulation with UVB for 30 min, 1 or 2 h. UVB-induced phosphorylation of ERK, JNK, and p38 MAPK was then determined by western blot analysis. IXA pre-treatment significantly inhibited the UVB-induced phosphorylation of ERK, JNK, and p38 MAPK in a dose-dependent manner without affecting the total protein levels of these kinases (Fig. 7). These results indicated that the inhibitory effect of IXA on UVB-induced MAPK phosphorylation may result in blockage of the cytokine production and COX-2 expression in HaCaT cells.

Discussion

Naturally occurring chemical substances derived from plants have been of much interest as therapeutics for several diseases. The species *Ixeris dentata* is a perennial herb of the Asteraceae family that is widely distributed and cultivated in northeastern Asia. The young sprouts of this species have been used as a famous bitter appetizing vegetable in Korea and also for the treatment of indigestion, pneumonia, hepatitis, contusions, and tumors (18).

Ixeris dentata is known to contain aliphatic compounds, triterpenoids, and sesquiterpene glycosides (19). Chemical components such as triterpenes, sesquiterpene glycosides, and flavonoids have been isolated from the genus *Ixeris*, encompassing ~20 species (28). IXA is an active constituent isolated from the extract of *Ixeris dentata*. Water and organic extracts of *Ixeris dentata* have shown strong hypolipidemic effects with antioxidant (20), antiallergic (21), monamine oxidase (22), anti-inflammatory (23), antimutagenic, and anticancer (24) activity. In particular, our previous studies have shown that EtOAc and tectroside isolated from *Ixeris dentata* showed protective effects against UVB-induced skin inflammation (25,26).

IXA was examined for its inhibitory effects on the human uterine carcinoma cell line, multidrug-resistant subline of MES-SA, human colorectal adenocarcinoma cell line, and multidrug-resistant subline of HCT15 according to the SRB assay *in vitro* (27).

However, to the best of our knowledge, no information is available concerning the effect of IXA on skin inflammation. In this study, we investigated the protective effect of IXA against UVB-induced damage in HaCaT cells. Keratinocytes are the main cells present in the epidermal layer of the skin and are exposed to a variety of stress stimuli such as UV radiation (1,2). UVB, in particular, has a wide spectrum of biological effects on the skin, and acute exposure causes a variety of adverse skin reactions such as erythema, edema, sunburn, hyperplasia, inflammation, and immunosuppression (7).

Cytokines such as IL-6 and IL-8 undoubtedly play pivotal roles in immunologic regulation in the human body and are involved in the induction of proliferation, differentiation, and cell death in many cell types such as leukocytes (29). These pro-inflammatory cytokines are considered to be

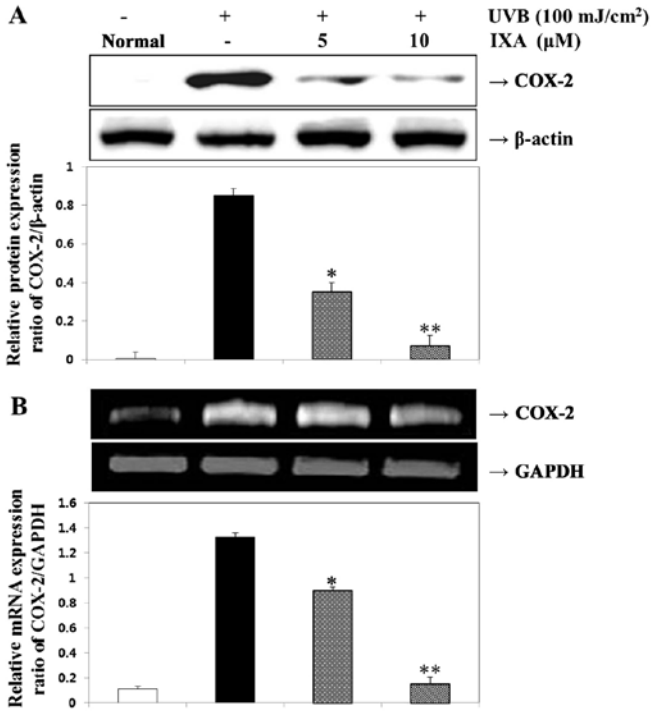

Figure 6. Effect of ixerisoside A (IXA) on ultraviolet B (UVB)-induced cyclooxygenase-2 (COX-2) protein and mRNA expression in human keratinocytes (HaCaT cells). HaCaT cells were pre-treated with the indicated concentrations of IXA for 24 h prior to being irradiated with UVB (100 mJ/cm^2) for 24 h. Equal amounts of protein (20 μg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with COX-2 antibodies. Equal protein loading was verified using β-actin. (A) COX-2 mRNAs were assessed by reverse transcription-polymerase chain reaction (RT-PCR) in HaCaT cells. The cells were pre-treated with the indicated concentrations of IXA for 24 h prior to being incubated with UVB (100 mJ/cm^2) for 24 h. (B) β-actin mRNA was assayed in parallel to confirm the equivalency of the cDNA preparations. The experiment was repeated three times, and similar results were obtained. *P<0.05, **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 means ± standard error (SE) of duplicate determinations from three separate experiments.

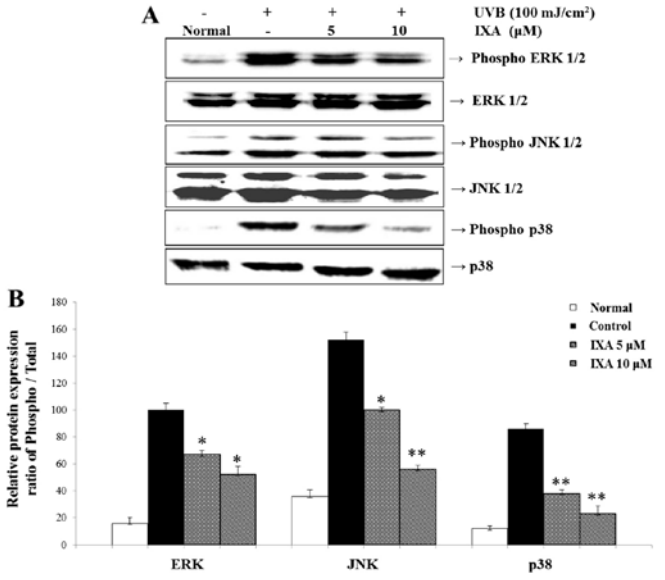

Figure 7. Effect of ixerisoside A (IXA) on the phosphorylation of mitogen-activated protein kinases (MAPKs) in ultraviolet B (UVB)-stimulated human keratinocytes (HaCaT) cells. (A and B) HaCaT cells were treated with the indicated concentrations of IXA for 24 h before being irradiated with UVB (100 mJ/cm^2) for 30 min, 1 and 2 h. Whole-cell lysates were analyzed by western blot analysis. The experiment was repeated three times and similar results were obtained. *P<0.05, **P<0.005.

closely associated with the progression of photodamage. In particular, IL-6 stimulates keratinocyte proliferation and is therefore studied in diseases associated with epidermal hyperplasia and in wound healing (30-34). IL-8, a powerful neutrophil attractant, is produced by keratinocytes after external stimuli such as arsenic, contact sensitizers, and irritants. IL-8-stimulated keratinocyte proliferation has been observed in auto-immune-mediated diseases such as pemphigus herpetiformis and bullous pemphigoid (35-38). Therefore, the inhibitory effect of IL-6 and IL-8 may have favorable anti-inflammatory effects on skin diseases. To test this hypothesis, we determined the inhibitory effect of IL-6 and IL-8 in UVB-irradiated HaCaT cells and demonstrated the suppression of UVB-induced intracellular production of IL-6 and IL-8. These results provide direct evidence to prove that IXA acted as an inhibitory agent for IL-6 and IL-8.

Moreover, COX-2 expression was induced by inflammatory cues in various tissues such as the epidermis (11,12). Previous studies have shown that chronic exposure to UVB radiation increases COX-2 expression through various cell signaling pathways, resulting in the induction of skin cancer (13,14). Previous findings have shown that UVB irradiation significantly increases COX-2 gene expression at the mRNA and protein levels in the human skin and cultured keratinocytes (14,39-41), and the resulting synthesis of prostaglandins with their growth-promoting and anti-apoptotic activities may be a contributory factor to UV radiation-induced skin carcinogenesis (42). Our results show that IXA inhibited UVB-induced COX-2 expression. Thus, we determined the effect of IXA and regulation of COX-2 in UVB-exposed keratinocytes.

UV irradiation activates the cell surface growth factor and cytokine receptors in fibroblasts, stimulating MAPK signal transduction pathways. ERK, JNK, and p38 are three families of MAPKs existing in mammalian cells, each of which forms a signaling module (43). MAPKs are a large family of protein kinases that phosphorylate and sequentially activate one another in a series of distinct cascades in response to markedly diverse sets of stimuli involved in the regulation of development, growth, differentiation, inflammation, and cell death (44). The expression of inflammatory cytokines (COX-2 and IL-6) has been shown to be affected by various intracellular signaling MAPK proteins such as ERK, JNK, and p38 (45). UVB exposure induces the rapid activation of MAPK signals such as p38 leading to COX-2 expression in HaCaT cells (15). Our results indicate that IXA inhibited UVB-induced MAPKs phosphorylation. Additionally, the anti-inflammatory effect of IXA inhibition of MAPK phosphorylation in keratinocytes exposed to UVB irradiation was confirmed.

We examined the effect of IXA on UVB-induced pro-inflammatory cytokine production in HaCaT cells by evaluating UVB-stimulated cells in the presence or absence of IXA. Pro-inflammatory cytokine production was measured

by ELISA and RT-PCR, and the activation of MAPKs was determined by western blot analysis. The inhibitory effects of IXA on the production of inflammatory mediators were accompanied by a concentration-dependent decrease in the protein and mRNA expression levels of IL-6, IL-8, and COX-2. Therefore, IXA most probably acted as an anti-inflammatory agent by mainly inhibiting COX-2 expression *in vivo*. Furthermore, these effects were mediated by the inhibition of COX-2 expression and ERK, JNK, and p38 MAPK phosphorylation.

These results indicate that the inhibitory effect of IXA on UVB-induced MAPK phosphorylation may result in blockage of the cytokine production and COX-2 expression in HaCaT cells. Therefore, this shows a potent anti-inflammatory effect of IXA accomplished by blocking inflammatory mediators. Our data indicate that IXA is a new source of potential drugs that may be used for the treatment of inflammatory diseases.

In conclusion, we evaluated the effect of IXA on skin inflammation *in vitro* and found that IXA has potential to attenuate UVB-induced skin inflammation by suppressing MAPK activation. Our findings provide new insight into the application of IXA and its nutraceutical value. Further studies on IXA are required to confirm its medicinal use.

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