Protection of Human Keratinocytes from UVB-Induced Inflammation Using Root Extract of Lithospermum erythrorhizon

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UVB irradiation is an important inducer of biological changes in skin and can activate inflammatory reactions and apoptotic pathways, leading to skin damage. A root extract of Lithospermum erythrorhizon (SK), which has naphthoquinone pigments containing shikonin and shikonin derivatives, is known for its anti-inflammatory, anti-bacterial, and anti-tumor activity, and for its scavenging of reactive oxygen species. However, the effect of SK against UV damage is not clear. The aim of this study was to evaluate the efficacy of SK against UVB induced damage in normal human epidermal keratinocytes (NHEK). UVB-irradiated NHEK showed decreased cell viability, increased production of interleukin (IL)-1α, IL-6, IL-8, and tumor necrosis factor-α, and induced apoptosis. In an apoptosis pathway assay, UVB-irradiated NHEK showed increased caspase-3 activity, p53 and its phosphorylation at serine 15 compared with non-irradiated cells. All these effects induced by UVB irradiation were clearly inhibited by treatment with SK before and after UVB irradiation for 24 h. It is suggested that SK can protect epidermal cells against harmful effects of UVB irradiation and that SK treatment is probably beneficial for photoprotection of the skin.

Key words  UVB; keratinocyte; Lithospermum erythrorhizon; inflammation; apoptosis

UVB irradiation is the major environmental cause of skin damage.1) UVB exposure of human skin induces skin alterations, including erythema,2) which is characteristic of sunburn cells,3) and prolonged UVB exposure results in the formation of wrinkles, the degradation of matrix molecules,4) the development of elastosis,5) and an increased risk of epithelial skin cancer.1,6,7) Keratinocytes are the main target of UV, and play a central role in several responses of photodamage after UV exposure by means of the release of pro-inflammatory cytokines such as interleukin (IL)-1α, IL-6, IL-8, and tumor necrosis factor (TNF)-α.7) IL-1 is a potent inducer of IL-6, and one may speculate that the release of IL-6 by keratinocytes after UV exposure is mediated by the release of IL-1 in an autocrine or paracrine manner.8) Induction of IL-10 and TNF-α by UV results in systemic immunosuppression,9) and IL-6 and IL-8 induce the acute-phase response and stimulates leukocyte infiltration in the skin.6) These pro-inflammatory cytokines are considered to be closely related to the progression of photodamage.

An additional process of photodamage is apoptosis in cells that contain a high proportion of damaged DNA following UV irradiation.12) The primary mediator responsible for removing UVB-induced DNA damage of keratinocytes in skin is believed to be p53,13–15) which activates downstream genes and subsequently induces cell-cycle arrest in the G1-S phase to repair DNA mutations.16) If the cells have excessive unrepaired DNA damage, a process that leads to fragmentation of the DNA is initiated and apoptotic sunburn cells are formed.17) In this way, apoptosis is considered to act as a crucial mechanism for the elimination of keratinocytes damaged by UV irradiation.17) In recent years it has been indicated that the molecular mechanisms of inflammation and apoptosis are similar,18,19) and this finding suggests that inflammation and apoptosis are related to the repair and recovery of UVB-induced cell and tissue damage.

To search for an effective compound to protect skin against these deleterious effects of UV, we examined the effects of various substances on human keratinocytes using inflammatory cytokine production and cell viability as indexes of photodamage. We examined various substances and found that the root extract of Lithospermum erythrorhizon (SK) suppresses UVB damage. SK contains naphthoquinone derivatives, which have been reported to have anti-inflammatory,20) anti-bacterial,21) and anti-tumor effects,22) and scavenging activity against several types of reactive oxygen species (ROS).23,24) However, the cellular and molecular mechanisms by which SK inhibits UVB-induced damage are not clear. In this study, we demonstrated that SK protects human keratinocytes from UVB-induced damage in vitro.

MATERIALS AND METHODS

Materials  SK was prepared from the root of Lithospermum erythrorhizon Sieb. et Zucc. (Koshiro Company, Osaka, Japan) by extraction with ether under reflux, and the component that the solvent removed was used in our studies. Acetylshikonin, isobutyrylshikonin, β,β-dimethylacyrshikonin, isovalerylshikonin, and α-methyl-n-butyrilshikonin were purchased from Nagara Science (Gifu, Japan). Shikonin, β-hydroxyisovalerylshikonin, and HPLC-grade methanol, acetonitrile, triethylamine, acetic acid, as well as phenylmethylsulfonyl fluoride, aprotinin, and leupeptin were purchased from Wako Pure Chemical Industries (Osaka, Japan). O,O-Bisethyleneglycol-N,N,N',N'-tetraacetic acid (EGTA) and ethylenediamine-N,N',N'-tetraacetic acid (EDTA) were obtained from Dojindo Laboratories (Kumamoto, Japan).

Cell Culture  Normal human epidermal keratinocytes (NHEK) were purchased from Kurabo (Osaka, Japan). NHEK were cultured in a serum-free keratinocyte growth medium (Kurabo) containing insulin (10 μg/ml), bovine pituitary extract (0.4% v/v), human recombinant epidermal growth factor (0.1 ng/ml), hydrocortisone (0.5 μg/ml), gentamicin (50 μg/ml), and amphotericin (50 ng/ml) in a humidified atmosphere with 5% CO2 at 37°C. NHEK were used at
UVB Irradiation and SK Treatment

The source of UVB irradiation was the Ultraviolet Transilluminator, Model NTFM-62 (UVP, Upland, CA, U.S.A.) equipped with two F25T8 UVB bulbs (single wavelength, 302 nm). UV dosimetry was performed with a Darmaray UV Meter and Detector (Gigahertz-Optik, Pochheim, Germany). NHEK were grown in 12-well plates (Corning, NY, U.S.A.) at 3×10^6 cells/ml to the subconfluence and preincubated with different concentrations of SK with keratinocyte basal media without supplements for 24 h. After pretreatment, media were removed and replaced with phosphate-buffered saline (PBS). The cells were exposed UVB (0—52.5 mJ/cm²) irradiation in the absence of SK without the plastic lid. Immediately after UVB irradiation, the irradiated cells were placed in fresh medium containing the same concentration of SK for 24 h.

Cell Viability Assay

Cell viability after UVB irradiation was analyzed by the neutral red assay. NHEK were incubated with neutral red-containing medium (50 µg/ml) for 2 h and then fixed in 1% formaldehyde and 1% CaCl₂ for 1 min, and lysed with 1% acetic acid and 50% ethanol for 20 min. Absorbance at 540 nm was measured with a microplate reader. Viability was calculated as 100% in absorption level of control.

Measurement of Inflammatory Cytokines

The cultured supernatants were collected after UVB irradiation for 3 h, 6 h, 12 h, and 24 h. IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α in cultured supernatants were determined with a Human Inflammation Cytometric Bead Array (CBA) Kit (Becton Dickinson, San Diego, CA, U.S.A.) according to the manufacturer's protocol. Samples were measured using FACSCalibur™ (Becton Dickinson) and sample results were analyzed in graphical and tabular format using the Becton Dickinson CBA analysis software. IL-1α was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer's instructions.

Morphologic Detection of Apoptosis

The cells were harvested following UVB irradiation for 24 h, and fixed with 1% (v/v) glutaraldehyde (Wako) in PBS for 30 min. After washing once with PBS, the cells were stained with 1 mM Hoechst 33258 (Wako) for 5 min in the dark. Chromatin condensation was examined using fluorescence microscopy. Experiments were performed at least in triplicate.

Apoptosis Assay by ELISA

The apoptosis assay was performed using ApoStand™ ELISA Apoptosis Detection Kit (Bio Mol, Plymouth Meeting, PA, U.S.A.) according to the manufacturer's protocol. This kit is based on the sensitivity of DNA in apoptotic cells to formamide denaturation and the detection of the denatured DNA with a monoclonal antibody to single-stranded DNA. The absorbance at 405 nm was measured using a microplate reader.

Quantification of p53 Protein by Western Blotting

UVB-irradiated cells were detached with 0.025% trypsin solution (Kurabo) and resuspended in lysis buffer (50 mM Tris–HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM sodium vanadate, 1 mM NaF, 10 µg/ml each of phenylmethylsulfonyl fluoride, aprotinin, and leupeptin). Protein concentration was determined using the Bicinchoninic Acid Protein Assay Kit (Pierce, Rockford, IL, U.S.A.). A quantity 75 µg of total protein extract was separated by 10% SDS-PAGE at 200 V and electrophotorecantly transferred onto the PVDF membrane (Bio-Rad, Hercules, CA, U.S.A.) using Semi-Dry Transblot apparatus (Bio-Rad) for 40 min at 25 mA. The membrane was washed in 0.1% Tween-20 in PBS (T-PBS) and blocked with 5% nonfat dry milk in T-PBS for 40 min at room temperature. The membrane was probed with 1 µg/ml of a goat anti-p53 antibody (R&D Systems) in T-PBS, incubated at 4°C overnight. After washing in T-PBS, blots were reacted with 0.2 µg/ml of HRP-conjugated rabbit anti-goat IgG (American Qualex, San Clemente, U.S.A.) in T-PBS. Protein bands were visualized using a chemiluminescent substrate (Pierce) and exposed to Kodak X-ray film.

Analysis of p53 Serine 15 by ELISA

The analysis of p53 serine 15 was performed using a DuoSet IC ELISA kit (R&D Systems) according to the manufacturer's protocol. A biotinylated detection antibody specific for human p53 phosphorylated at serine 15 in cell lysates was used to detect phosphorylated protein with a standard streptavidin-HRP method. The cell extracts were prepared by lysing the irradiated cells in buffer as above. The results were read at 450 nm.

Caspase Assay

At the indicated time points, NHEK after UVB irradiation were extracted in lysis buffer (50 mM Tris–HCl pH 7.4, 1 mM EDTA, 1 mM EGTA, and 0.2% (v/v) Triton X-100). The cell lysates were incubated at 37°C for 10 min and prepared by centrifugation at 1340 g for 10 min. The cleared lysates were incubated with 50 µM enzyme substrate Ac-ASP-Glu-Val-Asp-MCA, Ac-Leu-Glu-His-Asp-MCA, and Ac-Ile-Glu-Thr-Asp-MCA (Peptide Institute, Osaka, Japan) at 37°C for 1 h. Levels of released 7-amino-4-methylcoumarin were measured with a spectrophotometer (Hitachi F-4000, Ibaragi, Japan) with excitation at 380 nm and emission at 460 nm.

Inhibition of Apoptosis by Caspase Inhibitor

NHEK were treated with caspase-3 inhibitor (Ac-Asp-Gul-Val-Asp-H; Peptide Institute) in keratinocyte basal medium without supplements before and after UVB irradiation for 24 h. Cell viability after exposure to caspase-3 inhibitor was evaluated by neutral red assay as described above.

Analysis of Extract of SK by HPLC and Assay of Effects of Components of SK on Cell Viability

The compounds contained in SK were separated using a Waters 600 HPLC system (Waters, Milford, MA, U.S.A.). A Puresil™ C18 column (5 µm, 4.6×150 mm) (Waters) was used, at room temperature. The mobile phase was acetonitrile, water, triethylamine, acetic acid (70:30:0.3:0.3, v/v) and the flow rate was 0.8 ml per min. We tested the effects of seven components of SK on cell viability using the neutral red assay as described above.

Statistics

Data are presented as mean ± standard deviation (S.D.). Statistical significance was assessed using Student's t test.

RESULTS

SK Recovers Cell Viability in UVB-Irradiated Keratinocytes

The initial experiment was designed to characterize the viability of cells exposed to various doses of UVB irradiation from 0 to 52.5 mJ/cm². Cell viability was measured with the neutral red assay after UVB irradiation. The viability of UVB-irradiated cells decreased in a dose-depend-
ent manner. The 50% inhibitory dose of UVB irradiation for cell viability was 27 mJ/cm² (Fig. 1), and we used this UVB-dose in the following experiment.

Treatment with SK at concentrations up to 1 µg/ml had no effect on the viability of irradiated cells. SK in the concentration range 2.5—7.5 µg/ml increased the viability of UVB-irradiated cells by 15—30% compared with UVB-irradiated cells not treated with SK (Fig. 2A). This result indicates that the cytotoxic effect of UVB irradiation on NHEK restored the cells almost to the same viability as non-irradiated cells when SK was applied both before and after UVB exposure.

Using fluorescence microscopy, we investigated the effect of UVB irradiation on the morphological features of NHEK and the effect of SK in protecting the cells from UVB-induced injury. Treatment with UVB at 27 mJ/cm² induced marked changes in the morphology of cells, as seen in the squamous appearance and rounding of the cells. However, SK treatment resulted in recovery of a normal appearance in UVB-irradiated cells (Fig. 2B). This result indicates that pre- and post-treatment with SK protected NHEK cultures against the UV-induced damage described above.

**SK Inhibits the Production of Inflammatory Cytokines in UVB-Irradiated Keratinocytes** We examined the effect of SK on the production of inflammatory cytokines in UVB-irradiated cells using flow cytometry and ELISA. Firstly, we studied the production of IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α following UVB exposure. After UVB irradiation, NHEK showed a significant increase in the release of IL-1α, IL-1β, IL-6, IL-8, and TNF-α into the cell supernatant, starting at 12 h post-irradiation and increasing significantly at 24 h (Fig. 3). In contrast, induction of release of IL-10 and IL-12p70 was not detected in cells exposed to 27 mJ/cm² of UVB irradiation (data not shown). For cytokine release, we examined the effect of SK treatment for 24 h before and after UVB irradiation. SK significantly reduced the level of IL-6, IL-8, and TNF-α protein in UVB-irradiated cells at 12 h and 24 h after UVB irradiation (Fig. 3). In addition, SK significantly inhibited the level of IL-1α in irradiated cells at 24 h and IL-1β in irradiated cells at 12 h after UVB irradiation (Fig. 3). These data indicate that SK suppresses the production of IL-1α, IL-1β, IL-6, IL-8, and TNF-α after UVB irradiation.

**SK Inhibits UVB-Induced Apoptosis** To test the involvement of apoptosis in UVB-induced cell death, 24 h after UVB exposure NHEK were treated with Hoechst 33258. Following apoptosis-inducing insults, keratinocytes enter the so-called round-up phase, which is followed by DNA fragmen-
NHEK were treated with SK before and after UVB (27 mJ/cm²) irradiation for 24 h. (A) Apoptosis of NHEK following UVB irradiation was detected with Hoechst 33258. Arrows indicate apoptotic cell. (B) ELISA assay was performed 24 h post-UV irradiation. 

Fig. 5. Prevention of UVB-Induced Up-Regulation of p53 Expression and Level of Serine 15-Phosphorylated of p53 by SK

NHEK were grown in 100 mm dish at 6×10⁴ cells/ml to the subconfluent, pretreated with SK for 24 h, and irradiated by UVB. NHEK were harvested 0, 3, 6, 9, and 24 h after UVB irradiation. (A) p53 protein expression was analyzed by Western blot using monoclonal antibody of p53. (B) Level of serine 15-phosphorylated p53 was measured by ELISA.

Active Components of SK Improve Cell Viability after UVB Irradiation

To investigate the composition of SK, we analyzed it by HPLC a with Puresil™ C18 column. HPLC was used to determine the relative amounts of the components of SK using standard samples. As shown in Table 1, SK consisted of shikonin, acetylshikonin, isobutyrylshikonin, isovalerylshikonin, β,β-dimethylacylshikonin, β-hydroxyisovalerylshikonin, and α-methyl-n-butyrylshikonin, which are red naphthoquinone pigments. Isovalerylshikonin and α-methyl-n-butyrylshikonin had the same retention times; however, the combination ratio was unclear.

Next, we tested the effects of seven of the components SK on cell viability. Except for α-methyl-n-butyrylshikonin, all ingredients led to significant increases in viability at the concentrations tested (Fig. 8). In particular, shikonin, isobutyrylshikonin, β-hydroxyisovalerylshikonin, and isovalerylshikonin produced significant recovery of cell viability at levels below the combinational quantity of each component in SK as revealed by HPLC. Furthermore, recovery of cell viability by the mixtures of shikonin, isobutyrylshikonin, β-
hydroxyisovalerylshikonin, and isovalerylshikonin were the almost same as SK (data not shown).

DISCUSSION

UVB radiation of the skin, which is known to damage epidermal cells, contributes to pathological conditions that include epidermal photoaging and photocarcinogenesis. In the skin, a delicate balance should be maintained between keratinocyte proliferation and cell death to ensure terminal differentiation and cornification in an orderly manner that is coordinated throughout all layers of the epidermis. However, when this balance is disturbed by UVB irradiation, the cells cannot repair damage to their DNA. A process that leads to fragmentation of the DNA is initiated and apoptotic sunburn cells are formed. In this study we demonstrate that SK protects human keratinocytes from UVB-induced damage in vitro. SK effectively reduced cell death and apoptotic DNA cleavage after UVB radiation (Figs. 2, 4B). It has been reported that apoptosis by UVB in keratinocytes was related to several pathways, including those involving cytokines such as...
as TNF-α and Fas ligand and the proteases of the caspase family. The involvement of these mediators differs according to the cell type, UV dose, source of light, and so on. The present study showed that apoptosis induced by UVB in NHEK was related to caspase-3, but not caspase-8, and caspase-9 (Fig. 6). Apoptosis is mediated via either of two pathways, an intrinsic pathway in which cell death occurs through the deterioration of mitochondria, and an extrinsic pathway in which cell death occurs through a cascade of reactions via a receptor-mediated pathway. In the intrinsic pathway, caspase-9 is activated via distress signals sent from the mitochondria. In the extrinsic pathway, caspase-8 is activated via signals sent from death receptors. Both intrinsic and extrinsic pathways of apoptosis merge into a common pathway for apoptosis. Caspase-3 is activated in the common pathway and serves as a critical marker for apoptosis. We showed that activation of caspase-3 in NHEK cells by UVB irradiation was inhibited when the cells were treated with SK before and after UVB irradiation (Fig. 6). It is considered that SK suppresses apoptosis in NHEK by inhibiting the activation of caspase-3.

To investigate the protective effect of SK against UVB-induced apoptosis, we assessed p53 and its phosphorylation at serine 15 in NHEK. It has been reported that UVB-induced apoptosis is related to stabilization and functional activation of p53. Stabilization of p53 occurs in response to DNA damage through complex mechanisms, and one of which is the phosphorylation of p53 at the N- and C-terminal residues, especially at serine 15.34 In the present study we expressed the p53 protein and serine 15-phosphorylated p53 protein were increased by UVB exposure, and the expression both was suppressed by SK treatment (Fig. 5). It is suggested that suppression of apoptosis by SK may inhibit the stabilization of p53. It is known that p53 activates cytochrome c and p21 via target genes such as Bax, Bak, Noxa, and Puma, or induced growth arrest genes such as p21 and GADD45. The induction of p53 is associated with rather limited transcriptional target gene activation, with primary enhancement of p21 and Apaf-1; there is little or no change for several other p53 target genes, such as Bax, Bak, and GADD45. Although we did not examine the expression of p21 and Apaf-1, caspase-8 and caspase-9 were not activated by UVB irradiation. In addition, Bel-2, which acts as an apoptotic inhibitor in the intrinsic pathway, was not increased by SK treatment in NHEK at 3, 6, 9, and 24h after UVB irradiation (data not shown). It is considered that apoptosis induced by UVB in NHEK is not mediated by both intrinsic and extrinsic pathways of apoptosis.

Keratinocyte-derived cytokines play an important role in the pathogenesis of UVB-induced immunological and inflammatory reactions. The present study indicated that after UVB irradiation there is a significant increase in the release of IL-1α, IL-1β, IL-6, IL-8, and TNF-α in the NHEK supernatant. Treatment of NHEK with SK before and after UVB irradiation prevented the UVB damage that inhibited the production of IL-1α, IL-1β, IL-6, IL-8, and TNF-α (Fig. 3). Anti-inflammatory mechanisms of shikonin derivatives that have been reported include the inhibition of leukotriene B4 biosynthesis, suppression of mast cell degranulation, and protection of the vasculature, and blockade of chemokine ligands binding to CC chemokine receptor 1. Inhibition of the release of inflammatory cytokines by SK suggests that there is another possible mechanism in the anti-inflammatory action of SK.

SK contains several shikonin derivatives. The composition of these derivatives differed according to the area of production. To identify the active ingredient of SK, we analyzed SK by HPLC. The results showed that SK contained shikonin, β-hydroxyisovalerylshikonin, acetylshikonin, isobutyrylshikonin, β,β-dimethylacrylshikonin, α-methyl-n-butyrylshikonin, and isoaverelylshikonin (Table 1). We tested the protective effect of identified components on cell viability. Treatment with shikonin, β-hydroxyisovalerylshikonin, isobutyrylshikonin, and isoaverelylshikonin led to a significant recovery of cell viability at concentrations below their relative amounts in SK as revealed by HPLC, and these mixtures were recovered deceased cell viability by UVB irradiation same as SK (Fig. 8). As the recovery by active ingredient became clear, we considered that the protective effect of SK against UVB damage was because of the additive effect.

In conclusion, UVB irradiation of NHEK induced apoptosis, which was mediated by expression of p53, phosphorylation at serine 15, and activated effectors such as the caspase-3 present in NHEK. The production of inflammatory cytokines increased after UVB irradiation. Furthermore, our results indicate that SK, a root extract of Lithospermum erythrorhizon, inhibited the UVB-induced reactions mentioned above. Consequently, SK treatment may be beneficial in the photoprotection of the skin.


