# Decursin inhibits UVB-induced MMP expression in human dermal fibroblasts via regulation of nuclear factor-κB

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**Abstract.** Decursin, a coumarin compound, was originally isolated from the roots of *Angelica gigas* almost four decades ago, and it was found to exhibit cytotoxicity against various types of human cancer cells and anti-amnesic activity *in vivo* through the inhibition of AChE activity. However, the anti-skin photoaging effects of decursin have not been reported to date. In the present study, we investigated the inhibitory effects of decursin on the expression of matrix metalloproteinase (MMP)-1 and MMP-3 in human dermal fibroblast (HDF) cells. Western blot analysis and real-time PCR revealed that decursin inhibited the ultraviolet (UV)B-induced expression of MMP-1 and MMP-3 in a dose-dependent manner. Decursin significantly blocked the UVB-induced activation of nuclear factor-κB (NF-κB). However, decursin showed no effect on MAPK or AP-1 activity. In this study, decursin prevented the UVB-induced expression of MMPs via the inhibition of NF-κB activation. In conclusion, decursin may be a potential agent for the prevention and treatment of skin photoaging.

## Introduction

Skin aging can be divided into intrinsic (chronologic) aging, which is the process of senescence that affects all body organs and extrinsic aging (photoaging), which occurs due to exposure to environmental factors. An important factor that influences extrinsic aging is sunlight, particularly exposure to ultraviolet (UV)B irradiation, which causes photoaging. Chronic exposure of human skin to UVB radiation results in photoaging and induces the production of matrix metalloproteinases (MMPs) (1).

MMPs are responsible for the degradation of the collagenous extracellular matrix (ECM) in connective tissues (2). MMP-1 preferentially degrades fibrillar collagens, which maintain the tensile strength of fetal membranes. In contrast, MMP-3 degrades an extremely wide array of ECM substrates and can activate secreted zymogenic forms of other MMPs (3).

UV exposure is an important factor in photoaging. UV irradiation of cultured human dermal fibroblasts (HDFs) *in vitro* or human skin *in vivo* induces the production of MMPs (4-6). Excessive matrix degradation by UV-induced MMPs secreted by various types of cells (e.g., keratinocytes, fibroblasts and inflammatory cells) has been shown to contribute significantly to connective tissue damage that occurs during photoaging (7,8). UVB irradiation can induce MMP expression by activating transcription factors, such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) (9,10). The mitogen-activated protein kinase (MAPK) signaling pathway is important for AP-1 activation; IκB kinase (IKK), phosphoinositide 3 kinase (PI3K)-Akt and p38 MAPK have been shown to activate NF-κB, depending on the cell type (11,12). Thus, the inhibition of UVB-induced MMP expression and/or its upstream regulatory pathways is critical for the treatment of photoaging of the skin.

Decursin is a coumarin compound found in the roots of *Angelica gigas* Nakai, which has been traditionally used in Korean folk medicine as a tonic and for the treatment of anemia and other diseases (13). Decursin induces cell cycle arrest and apoptosis in human prostate, breast, bladder and colon cancer cells (14-16). Recent reports have demonstrated

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that decursin blocks MMP-9 expression through the inhibition of NF-κB activation in macrophages and cancer cells (17-19). However, the inhibitory effects of UVB-induced MMP expression through NF-κB activation by decursin are not yet well defined.

In the present study, we evaluated the preventive effects of decursin on the UVB-induced production of MMPs in HDFs. Decursin blocked the UVB-induced NF-κB pathway, which inhibits the expression of MMPs. These results suggest that decursin is useful for the prevention of skin photoaging.

## Materials and methods

*Materials*. 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and anti-β-actin antibody were purchased from Sigma (St. Louis, MO, USA). Primary antibodies for MMP-1 and MMP-3 were obtained from R&D Systems (Minneapolis, MN, USA). Dulbecco's modified Eagle's medium (DMEM) with high glucose level, Medium 154, growth supplement, fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were obtained from Gibco-BRL (Gaithersburg, ME, USA). Primary antibodies for p50, p65, IκBα, proliferating cell nuclear antigen (PCNA) and horseradish peroxidase (HRP)-conjugated IgG were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

*Plant extracts and purification*. The roots of *A. gigas* Nakai (Umbelliferae family) were extracted serially with methanol, ethylacetate and n-butanol and fractionated. From the ethyl-acetate fraction, decursin was isolated using silica gel column chromatography. After column chromatography, the structure of the purified coumarin compounds, decursin ($C_{19}H_{20}O_5$) and decursinol angelate (molecular weight, 328 g) were characterized by gas chromatography (Shimadzu, Kyoto, Japan), nuclear magnetic resonance (JEOL JNM-LA 400; Japan) and mass spectroscopy (JEOL-AX 505WA) at Daegu Haany University, Daegu, Korea.

*Isolation and culture of HDFs*. HDFs were aseptically isolated from foreskin. The epidermis and dermis were separated by incubation in media with 0.9 U/ml dispase at 4˚C for 16 h. After the epidermis and dermis were mechanically separated, the dermis was minced, attached to the surface of a tissue culture flask and incubated with DMEM containing 10% FBS for 1-2 weeks (20). Dermal fibroblasts that spread as radial outgrowths from the attached pieces of dermis were cultured in DMEM containing 10% FBS and 1% antibiotics at 37˚C in a 5% $CO_2$ incubator.

*UV irradiation*. HDFs were rinsed twice with PBS and irradiated using a UVB cross-linker (6x8 W, 312 nm; Model CL-508M; Vilber Lourmat, Paris, France) (20). HEKn were irradiated using a Stratalinker UV crosslinker (Model 2400; Agilent Technologies, Cold Spring, NY, USA). Immediately after irradiation, fresh serum-free medium was added to the HDFs, and complete growth medium was added to the HEKn. Responses were measured after incubation for each experimental condition. The same schedule of medium changes was followed for control cells.

*Determination of cell viability*. The protective effect of decursin against UV-induced cytotoxicity of HDFs was determined using the MTT assay. Briefly, HDFs were seeded at a density of $3x10^4$ cells/plate and allowed to attach. After 24 h, the cells were treated with various concentrations of decursin (1, 5, 10, 30 and 50 μM). After incubation for 24 h, the cells were washed twice with PBS and MTT (0.5 mg/ml PBS) was added to each well. The plates were incubated at 37˚C for 30 min. Formazan crystals that had formed were dissolved by adding DMSO (100 μl/well) and the absorbance was measured at 570 nm using a microplate reader (Model 3550; Bio-Rad, Richmond, CA, USA).

*Trypan blue exclusion test for cytotoxicity*. Cells were seeded onto a 10-cm dish and allowed to attach for 24 h. They were then treated with UVB at 25 mJ/cm$^2$. After 24 h, the cells were detached from the wells by treatment with trypsin, followed by staining with trypan blue; non stained cells were counted under an optical microscope with a hemocytometer.

*Western blot analysis*. HDFs ($2x10^6$ cells) were irradiated with UVB (25 or 15 mJ/cm$^2$); the cells were treated with decursin for 24 h and lysed using 40 μl of ice cold M-PER® Mammalian Protein Extraction Reagent (Pierce Biotechnology, Inc., Rockford, IL, USA). Protein concentrations in the lysates were determined using the Bradford method (21). Samples were separated using 10% SDS-PAGE gels with 3% stacking gels; the resolved proteins were transferred to a Hybond™-PVDF membrane using a western blot apparatus (Bio-Rad). Polyvinylidene fluoride (PVDF) membranes were blotted with 1 μg/ml of primary antibodies for MMP-1, MMP-3, p50, p65, PCNA, or β-actin. HRP-conjugated IgG was used as a secondary antibody. Protein expression levels were determined by analyzing the signals captured on the PVDF membranes using an image analyzer (LAS-1000; Fuji Film, Japan).

*Quantitative real-time PCR assay*. Total RNA was extracted from cells using a FastPure™ RNA kit (Takara Bio, Inc., Shiga, Japan). RNA concentration and purity were determined by measuring the absorbance at both 260 and 280 nm. Then, cDNA was synthesized from 1 μg of total RNA using a PrimeScript™ RT reagent kit (Takara Bio, Inc.). MMP-1 and MMP-3 mRNA expression levels were analyzed using real-time PCR with the ABI PRISM 7900 sequence detection system and the SYBR-Green reagent (Applied Biosystems, Foster City, CA, USA). Primers used in the reaction were:MMP-1 (NM 002424.2) sense,5'-AGTGACTG-GGAAACCGATGCTGA-3' and antisense, 5'-CTCTTG-GCAAATCTGGCCTGTAA-3'; MMP-3 (NM 002422) sense, 5'-ATTCCATGGAGCCAGGCTTTC-3' and antisense, 5'-CATTTGGGTCAAACTCCAACTGTG-3' and GAPDH (NM 002046) sense, 5'-ATGGAAATCCC ATCACCATCTT-3' and antisense, 5'-CGCCCCACTTGA TTTTGG-3'. To control for variations in the mRNA concentration, all results were normalized to the housekeeping gene GAPDH. Relative

quantitations were performed using the comparative ΔΔCt method according to the manufacturer's instructions.

*Determination of MMP-1 and MMP-3 secretion with ELISA*. HDFs were seeded in 100-mm culture dishes at a density of $2x10^6$ cells/dish and then irradiated with UVB (25 mJ/cm$^2$). Following 24 h of incubation, the culture supernatants were collected and centrifuged at 10,000 x g for 5 min to remove the particulate matter and stored at -80˚C in fresh tubes. The protein concentration in the supernatants was determined using the Bradford method (21). The active MMP-1 in culture supernatants was quantified by fluorescent assay, using the Fluorokine E Human Active MMP-1 Fluorescent assay kit, and MMP-3 in the cell culture supernatants was then determined using Quantikine ELISA kits (all from R&D Systems), according to the manufacturer's protocol.

*Preparation of nuclear extract*. HDFs ($2x10^6$ cells) were irradiated with 25 mJ/cm$^2$ UVB and then treated with decursin for 3 h. Cells were immediately washed twice, scraped into 1.5 ml of ice cold PBS (pH 7.9) and then pelleted at 12,000 x g for 30 sec. Cytoplasmic and nuclear extracts were prepared from cells using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology).

*Electrophoretic mobility shift assay (EMSA)*. Activation of NF-κB and AP-1 was assayed with a gel mobility shift assay using nuclear extracts. An oligonucleotide containing the κ-chain (κB, 5'-CCGGTTAACAGAGGGGGCTTTCCGAG-3') or AP-1 (5'-CGCTTGATGAGTCAGCCGGAA-3') binding site was synthesized and used as a probe for the gel retardation assay. The two complementary strands were annealed and labeled with [α-32P]dCTP. Labeled oligonucleotides (10,000 cpm), 10 μg of nuclear extracts and binding buffer [10 mM Tris-HCl (pH 7.6), 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng poly(dI·dC), 1 mM dithiothreitol] were then incubated for 30 min at room temperature in a final volume of 20 μl. The reaction mixtures were analyzed by electrophoresis on 4% polyacrylamide gels in 0.5X Tris-borate buffer. The gels were dried and examined by autoradiography. Specific binding was controlled by competition with a 50-fold excess and cold AP-1 oligonucleotide.

*Statistical analysis*. Statistical analysis was performed using analysis of variance (ANOVA) and Duncan's test. A P-value <0.05 was considered to indicate a statistically significant result.

## Results

*Decursin protects HDFs against UVB irradiation*. The structure of decursin is shown in Fig. 1A. To investigate the cytotoxicity of decursin, HDFs were treated with various concentrations of decursin for 24 h. Cell viability was determined using the MTT assay. Decursin did not cause a significant change in the viability of HDFs up to 50 μM (Fig. 1B). To investigate the cell protective effect of decursin on UVB-induced cytotoxicity, cells were incubated with the indicated concentrations of decursin for 24 h in the presence of UVB. UVB-induced cytotoxicity was determined using the trypan blue exclusion test. Decursin (10 and 30 μM) significantly inhibited cell toxicity induced by UVB irradiation (Fig. 1C).

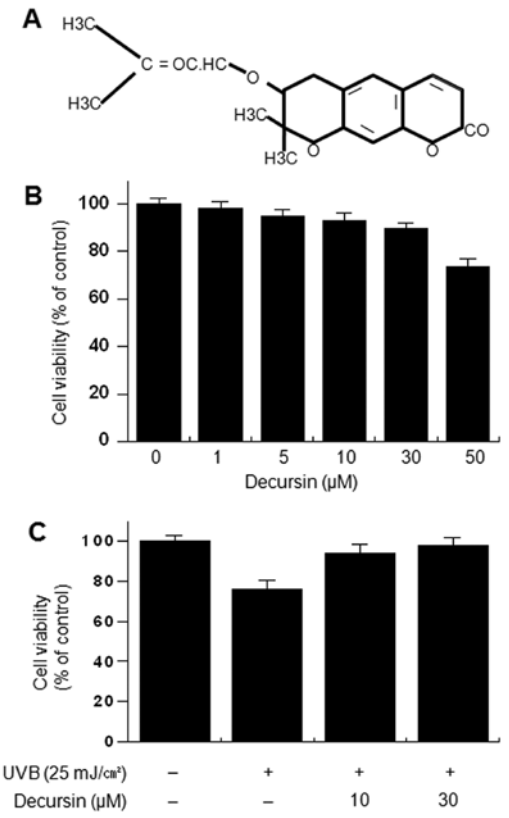


Figure 1. Structure of decursin and the effect of decursin on the viability of HDFs. (A) Chemical structure of decursin. (B) HDFs were cultured in 96-well plates until reaching 70% confluence, and various concentrations of decursin were added to the cells for 24 h. An MTT assay was used to detect cell viability. (C) Cells were cultured in 100-mm culture dishes until reaching 70% confluence and incubated with the indicated concentrations of decursin for 24 h under UVB irradiation. Cell viability was determined by cell counting. The optical density value of control cells was normalized to 100%. Data represent the means ± SEM of 3 independent experiments.

*Decursin inhibits the UVB-induced expression and secretion of MMP-1 and MMP-3 in HDFs*. UVB activates MMP secretion, which is a hallmark of skin aging (4,5,22). We examined the effects of decursin on UVB-induced expression of MMP-1 and MMP-3. Western blot analysis revealed that irradiation of HDFs with UVB (25 mJ/cm$^2$) markedly increased MMP-1 and MMP-3 levels (Fig. 2A). The UVB-induced increase in MMP levels was significantly reduced by treatment with decursin. Consistent with these results, real-time PCR analysis also showed an increase in expression of MMP-1 and MMP-3 mRNA after UVB irradiation while treatment of HDFs with decursin suppressed this UVB-induced increase in MMP-1 and MMP-3 expression (Fig. 2B). We also determined the effect of decursin on UVB-induced MMP secretion with ELISA. UVB irradiation of HDFs resulted in an increase in MMP-1 and MMP-3 secretion, while decursin significantly diminished the UVB-induced MMP-1 and MMP-3 secretion (Fig. 2C). Decursin itself had no effects on expression and secretion of MMP-1 and MMP-3 in HDFs. These results

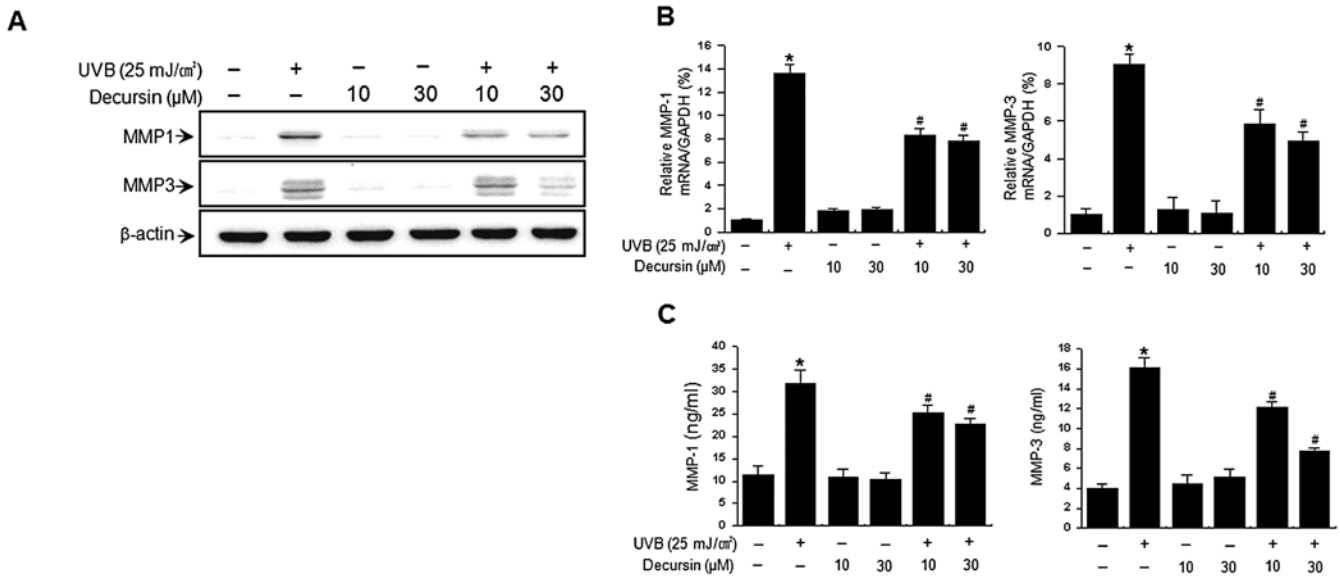


Figure 2. Effect of decursin on UVB-induced MMP-1 and MMP-3 expression in HDFs. Cells were stimulated with UVB (25 mJ/cm$^2$) and incubated with the indicated concentrations of decursin for 24 h. (A) Cell lysates were analyzed by western blot analysis with anti-MMP-1 and -MMP-3 antibodies. (B) Total cellular RNA was analyzed using real-time PCR for MMP-1 and MMP-3. (C) The presence of MMP-1 and MMP-3 in cell-free culture supernatants was measured using a commercially available ELISA kit as described in Materials and methods. Each value represents the means ± SEM of 3 independent experiments. $^*$P<0.01 vs. untreated control; $^\#$P<0.01 vs. UVB.

indicate that decursin inhibits the UVB-induced expression and secretion of MMP-1 and MMP-3 in HDFs.

*Effect of decursin on UVB-induced NF-κB and AP-1 DNA-binding activities.* To clarify the mechanism of decursin-mediated inhibition of MMP-1 and MMP-3 expression, the effect of decursin on UVB-induced activation of NF-κB and AP-1 was evaluated using EMSA and western blot analysis. As shown in Fig. 3A and B, pre-treatment with decursin inhibited UVB-induced DNA binding activity of NF-κB, but not AP-1. Decursin itself had no effect on the DNA binding activity of NF-κB or AP-1. Additionally, we determined the levels of p65, p50, p-c-Jun in the nuclear fraction. Cell treatment with UVB resulted in increased levels of p65, p50 and p-c-Jun; however, decursin blocked the UVB-induced translocation of p65 and p50 to the nucleus (Fig. 3C). These results suggest that decursin specifically blocks NF-κB activation in HDFs. The IκB kinase (IKK) enzyme complex is part of the signal transduction cascade upstream of NF-κB. IKK specifically phosphorylates the inhibitory IκB protein. Under basal conditions, the cytoplasmic protein IκB directly binds to p65 and p50 subunits and represses their nuclear translocation. IKK phosphorylation results in the dissociation of IκB from NF-κB and thereby activates NF-κB (23-26). Therefore, we determined the changes in the levels of p-IKKαβ and p-IκBα in the cytoplasmic fraction. The cytoplasmic fraction of UVB-stimulated HDFs showed higher levels of p-IKKαβ and p-IκBα than in unstimulated cells; however, the UVB-induced increase in the levels of p-IKKαβ and p-IκBα was significantly suppressed by treatment with decursin (Fig. 3D).

*Effect of decursin on the UVB-induced MAP kinase signaling pathway.* Since MAP kinase is an upstream regulator of NF-κB and AP-1, the role of MAP kinase (ERK, p38 and JNK) in the activation of MMP expression is fairly well understood (27,28). We investigated the effect of decursin on UVB-induced activation of MAP kinase. Decursin showed no effects on MAPK (Fig. 4). These results suggest that the MAPK pathway is not involved in the regulation of UVB-induced expression of MMP by decursin.

## Discussion

In the present study, we demonstrated the preventive effects of decursin on photoaging caused by MMP-1 and MMP-3. In previous studies, decursin was found to prevent MMP-9 expression by suppression of the NF-κB pathway in cancer cells and macrophages (17-19). Our results also demonstrated that decursin blocked UVB-induced activation of NF-κB, which has an important role in MMP-1 and MMP-3 expression.

Skin aging can be attributed to extrinsic aging (photoaging) and intrinsic (chronological) aging. Photoaging involves premature skin aging caused by repeated exposure to the sun (8,29,30). UV irradiation of cultured HDFs *in vitro* or human skin *in vivo* was found to induce the expression of MMP-1 and MMP-3, which play important roles in ECM components during skin aging (5,6,31). Varani *et al* (32) reported that MMP levels increase and collagen synthesis decreases in sun-protected human skin *in vivo* as age increases. Moreover, it was suggested that excessive matrix degradation by UV-induced MMPs secreted by various types of cells (e.g., keratinocytes, fibroblasts and inflammatory cells) contributes substantially to connective tissue damage that occurs during skin photoaging (7,8,33). Thus, we focused on the targets of decursin's action in signal transduction pathways involved in the induction of the two major MMP family members after UVB irradiation.

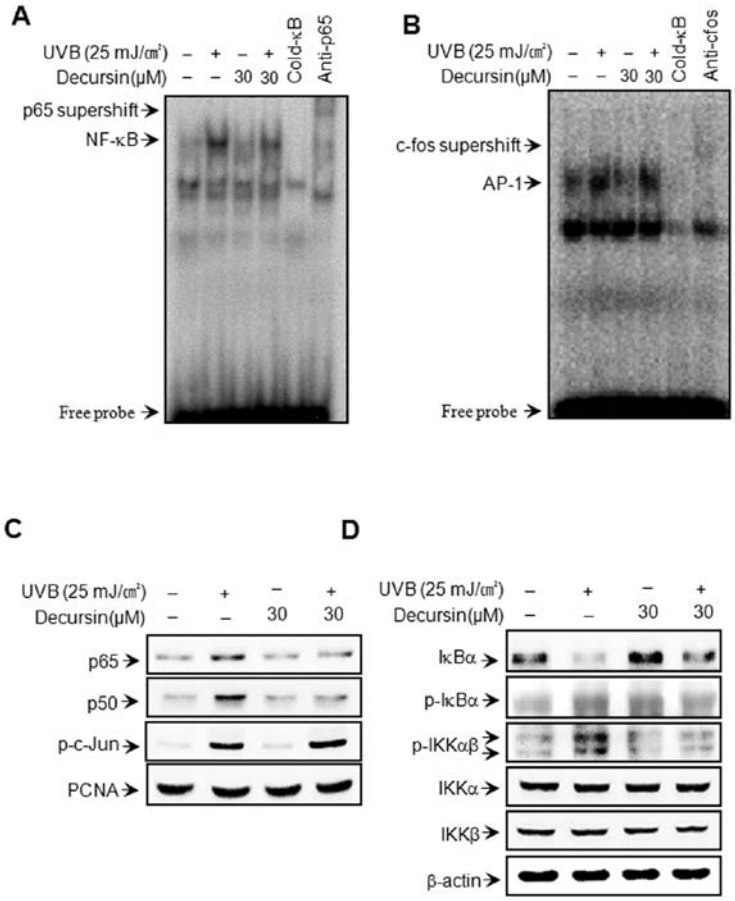


Figure 3. Decursin blocks UVB-induced NF-κB activation in HDFs. Cells were stimulated with UVB (25 mJ/cm$^2$) and incubated with the indicated concentrations of decursin. (A and B) Following 3 h of incubation, nuclear extracts were prepared. NF-κB and AP-1 DNA binding was analyzed using electrophoretic mobility shift analysis as described in Materials and methods. (C and D) Western blotting was performed to determine the nuclear levels of NF-κB (p50 and p65) and AP-1 (p-c-Jun) subunits, as well as the cytoplasmic levels of p-IKKαβ, IKKα, IKKβ, p-IκBα and IκBα.

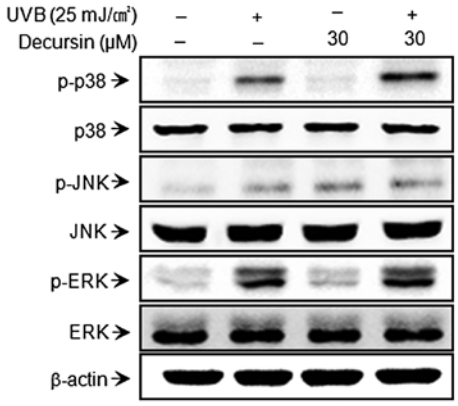


Figure 4. Effect of decursin on UVB-induced MAPK signaling activation in HDFs. Cells were pre-treated with UVB (25 mJ/cm$^2$) for 15 min in the presence or absence of decursin. Cell lysates were prepared for western blotting with antibodies against p38, JNK and ERK, as well as with phospho-specific antibodies for these antigens (p-p38, p-JNK and p-ERK).

UV irradiation includes three types: UVA (wavelength, 320-400 nm), UVB (280-320 nm) and UVC (200-280 nm). In particular, studies concerning skin have focused on UVB intensity due to stratospheric ozone depletion (34,35). It is well known that the UVB-inducible genes involved in skin aging are primarily composed of several MMPs involved in the degradation of the connective tissues of the skin (22,35). Recent studies have focused on the regulatory molecular mechanisms underlying UVB-induced upregulation of MMPs (36,37).

The present study found that transcription factors may be targets of decursin during UV-induced skin damage. NF-κB and AP-1 are ubiquitous transcription factors that govern the expression of genes encoding cytokines, chemokines, growth factors, cell adhesion molecules and several acute phase proteins in healthy and disease states (38,39). Therefore, the development of strategies that target these transcription factors may provide novel therapeutic tools for treating or preventing various diseases. UVB-mediated photoaging is prevented by the suppression of NF-κB and AP-1 activation (31,40,41). In fact, NF-κB and AP-1 are known to increase MMP-1 expression in the dermis (42,43). These studies suggest that NF-κB and AP-1 play important roles in MMP expression after UV irradiation. Previous studies demonstrated that NF-κB and AP-1 are molecular targets in decursin-treated cells (44,45), which suggests that targeting NF-κB and AP-1 in UV irradiation-mediated MMP expression by using decursin may provide a novel therapeutic tool

for treating or preventing photoaging. Our results showed that decursin strongly blocked UVB-induced NF-κB activation (Fig. 3). Furthermore, decursin significantly inhibited UVB-induced expression of MMPs in HDFs (Fig. 2). Our data indicate that decursin is a potent inhibitor of UVB-mediated NF-κB activation, which blocks the UVB-induced expression of MMPs in HDFs.

The MAPK pathway is involved in the regulation of cell proliferation, apoptosis, cytokine expression and MMP production. The three major MAPK families, JNK, ERK and p38 kinase, are expressed in HDFs, and the active phosphorylated forms can also be detected (46,47). Previous studies have shown that the MAPK signaling pathway is important for AP-1 activation; I-κB kinase (IKK), phosphoinositide 3 kinase (PI3K)-Akt and p38 MAPK have been shown to activate NF-κB, depending on the cell type (11,12,48-50). In this study, decursin displayed no effects on phosphorylation of p38, JNK and ERK. These data indicate that decursin is involved in NF-κB, but not in the MAPK signaling pathway in HDFs

In conclusion, the development of novel MMP inhibitors may be a promising strategy for skin cancer therapy and photoaging. Our results demonstrate that decursin is a potent inhibitor of UVB-induced expression of MMPs that blocks the NF-κB signaling pathway in HDFs. Therefore, decursin may be a potential therapeutic candidate for the prevention and treatment of photoaging.

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