SPANDIDOS PUBLICATIONS

Radix clematidis extract inhibits UVB-induced MMP expression by suppressing the NF-κB pathway in human dermal fibroblasts

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Received December 22, 2008; Accepted February 10, 2009

DOI: 10.3892/ijmm_00000180

Abstract. Ultraviolet (UV)B irradiation induces the production of matrix metalloproteinases (MMPs), which are responsible for the degradation of collagenous extracellular matrix in connective tissues, causing skin photoaging. Although *Radix clematidis* is commonly used in Chinese medicine for the treatment of arthralgia, the anti-skin photoaging effects of *Radix clematidis* have not yet been reported. In the present study, we investigated the inhibitory effects of *Radix clematidis* extract (RCE) on MMP-1 and -3 expression of human dermal fibroblast cells via various *in vitro* experiments and elucidated the pathways of inhibition. Western blot analysis and real-time PCR revealed RCE inhibited UVB-induced MMP-1 and -3 expressions in a dose-dependent manner. UVB strongly activated nuclear factor-κB (NF-κB) activity, which was determined by IκBα degradation, nuclear localization of p50 and p65 subunit, and NF-κB binding activity. However, UVB-induced NF-κB activation was completely blocked by RCE pretreatment. These findings suggest that RCE prevents UVB-induced MMP expression through inhibition of NF-κB activation. In conclusion, RCE is a potential agent for the prevention and treatment of skin photoaging.

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Key words: Radix clematidis, matrix metalloproteinases, ultraviolet B, NF-κB, photoaging

Introduction

Skin aging is divided into intrinsic or chronologic aging, which is the process of senescence that affects all body organs, and extrinsic aging (photoaging), which occurs as a consequence of exposure to environmental factors. One of the most important extrinsic aging factors is sunlight, particularly exposure to ultraviolet (UV)B irradiation, which causes skin photoaging. The skin photoaging process increases skin fragility, laxity, roughness, dryness, pigmentation, blister formation, leathery appearance and formation of wrinkles (1). Chronic exposure of human skin to UVB radiation results in photoaging and induces the production of matrix metalloproteinases (MMPs) (2).

MMPs are responsible for the degradation or synthesis inhibition of collagenous extracellular matrix in connective tissues (3). Collagen represents the main component of the extracellular matrix of dermal connective tissue, and its concentration decreases in chrono- and photoaging. MMP-1 preferentially degrades fibrillar collagens, which maintain the tensile strength of fetal membranes, whereas MMP-3 degrades an extremely broad array of extracellular matrix substrates and activates the secreted, zymogenic form of other MMPs (4-6). UVB is known to induce the expression of interstitial collatinase (MMP-1) and stromelysin-1 (MMP-3) in human dermal fibroblasts (HDF) and normal human epidermis *in vivo* (7-9). Among those MMPs, MMP-1 is the most important MMP in the degradation of the extracellular matrix by skin photoaging (10).

Radix clematidis is the name given to the root of *Clematis florida* T_{HUNB}, a member of the *Ranuncluaceae* family. *Radix clematidis* is commonly used in traditional Chinese medicine to alleviate joint pain and relieve swelling. In recent studies,

Radix clematidis was reported to exert inhibitory effects against ß-cell damage by suppressing the NF-κB pathway and depigmentation of melanocytes (11,12).

In the present study, we evaluated the preventive effects of *Radix clematidis* extract (RCE) on UVB-induced MMPs expression in HDF. RCE was found to block the UVB-induced NF-κB pathway, thereby inhibiting the MMPs expression. These results indicate that *Radix clematidis* is useful as an anti-skin photoaging agent.

Materials and methods

Materials. Primary antibodies for MMP-1 and -3 were obtained from R&D Systems (Minneapolis, MN, USA). High glucose-containing Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were obtained from Gibco-BRL (Gaithersburg, MD, USA). 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and anti-ß-actin were obtained from Sigma. Antibodies of p50, p65, and IκBα, PCNA, and horseradish peroxidase (HRP)-conjugated IgG were from Santa Cruz Biochemicals (Santa Cruz, CA, USA).

Preparation of Radix clematidis extract. *Radix clematidis* was obtained from Wonkwang Oriental Medical Hospital in Iksan, Jeonbuk, Korea and its identity was confirmed by Ho-Joon Song, keeper of the Herbarium. Voucher samples were preserved for reference in the Herbarium of the Department of Physiology, School of Oriental Medicine, Wonkwang University (Omcphy, 2005-58). *Radix clematidis* (200 g) were then ground and extracted in boiling water for 4 h. The extract was centrifuged at 3,000 x g for 20 min, after which the supernatant was concentrated to 200 ml under reduced pressure and then freeze dried to 20.4 g. The sterile extract was then stored at -70˚C until use.

Isolation and culture of HDF. HDF was aseptically isolated from a circumcised neonatal foreskin. The epidermis and dermis were separated by incubation in 0.9 units/ml dispase in medium for 16 h at 4˚C. After the epidermis and dermis were mechanically separated, the dermis was minced and attached on the surface of a tissue culture flask and fed with DMEM containing 10% FBS for 1-2 weeks. The dermal fibroblasts spread as radial outgrowth from attached pieces of dermis, cultured in DMEM with 10% FBS.

UV irradiation. For UVB irradiation, we used UVB cross-linker (6x8 W, 312 nm, Model CL-508M, Vilber lourmat, Paris, France). In brief, serum-starved confluent cells were rinsed twice with PBS, and all irradiations were performed under a thin layer of PBS. Immediately after irradiation, fresh serum-free medium was added to the cells. Responses were measured after an incubation period of 24 h. Mock-irradiated controls followed the same schedule of medium changes without UVB irradiation.

Determination of cell viability. The effect of RCE on cell viability of HDF was determined using MTT assay. Briefly, $2x10^4$ cells/well were treated with various concentrations of RCE. After incubation for 24 h, cells were washed twice with PBS, MTT (0.5 mg/ml PBS) was added to each well and incubated at 37˚C for 30 min. The formazan crystals formed were then dissolved by adding DMSO (100 µl/well) at 570 nm using a microplate reader (Model 3550, Bio-Rad, Richmond, CA, USA).

Western blot analysis. HDF ($2x10^6$ cells) was irradiated with UVB (25 mJ/cm^2) and then treated with RCE for 24 h. Cells were lysed with 40 µl of ice-cold M-PER® Mammalian Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL). In lysates, the protein concentration was determined using the Bradford method (13). Samples were separated by SDS-PAGE with 10% acrylamide running and 3% acrylamide stacking gels, and then transferred to Hybond™ -PVDF membranes using a Western blot apparatus. The PVDF membranes were blotted with 1 µg/ml of primary antibodies for MMP-1, -3, p50, p65, PCNA and ß-actin. HRP-conjugated IgG was used as a secondary antibody. The protein expression levels were then determined by analyzing the signals captured on the PVDF membranes using an image analyzer (Las-1000, Fujifilm, Japan).

Quantitative real-time PCR assay. Total RNA was extracted from the cells using FastPure™ RNA Kit (Takara, Shiga, Japan). The concentration and purity of RNA were determined by absorbance at 260/280 nm. cDNA was synthesized from 1 µg total RNA using PrimeScript™ RT reagent Kit (Takara). The expression of MMP-1 and -3 mRNA was determined by real-time PCR using the ABI PRISM 7900 sequence detection system and the SYBR® Green (Applied Biosystems, Foster City, CA, USA). The primers were, MMP-1 (NM 002424.2) sense, AGTGACTGGGAAACCAGATGCTGA; antisense, GCTCTTGGCAAATCTGGCCTGTAA and MMP-3 (NM 002422) sense, ATTCCATGGAGCCAGGCTTTC; antisense, CATTTGGGTCAAACTCCAACTGTG and GAPDH (NM 002046) sense, ATGGAAATCCCATCACCA TCTT; antisense, CGCCCCACTTGATTTTGG. To control variation in mRNA concentration, all results were normalized to the housekeeping gene, GAPDH. Relative quantitation was performed using the comparative $\Delta\Delta C_t$ method according to the manufacture's instructions.

Determination of MMP-1 and -3 secretions by ELISA. HDF was seeded in 100 mm culture dishes at a density of $2x10^6$ cells per dish, and then irradiated with UVB (25 mJ/cm^2) and then treated with RCE. Following 24 h of incubation, the culture supernatant was 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 active MMP-1 in culture supernatants was quantified by fluorescent assay, using the Fluorokine E Human Active MMP-1 Fluorescent Assay Kit (R&D Systems) and MMP-3 in the cell culture supernatants were then determined using Quantikine ELISA kits (R&D Systems), according to the manufacturer's protocol.

Preparation of nuclear extract. HDF ($2x10^6$ cells) was irradiated with UVB (25 mJ/cm^2) and then treated with RCE for 24 h. Cells were immediately washed twice, scraped into 1.5 ml of ice-cold PBS (pH 7.4), and then pelleted at 12,000 x g for 30 sec. Cytoplasmic and nuclear extracts were

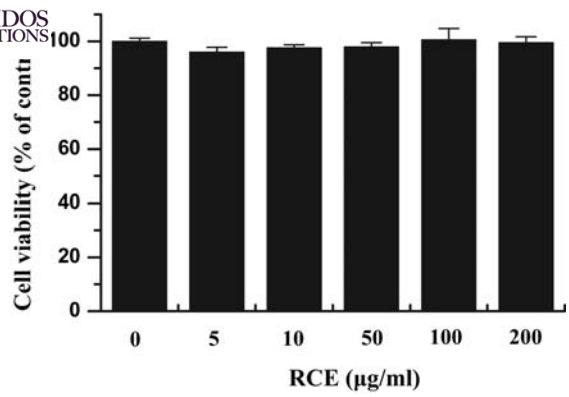

Figure 1. Effect of RCE on cell viability in HDF. Cells were cultured in 96-well plates until 70% confluence, then incubated with the indicated concentration of RCE for 24 h. MTT assay was used to detect the viability of cells as detailed in Materials and methods. The optical density value of control was regarded as 100%. Data points are the mean ±SE of three independent experiments.

prepared from cells using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology).

Electrophoretic mobility shift assay (EMSA). The activation of NF-κB was assayed by a gel mobility shift assay using nuclear extracts. An oligonucleotide containing the κ-chain binding site (κB, 5'-CCGGTTAACAGAGGGGGCTTTCCGAG-3') was synthesized and used as a probe for the gel retardation assay. The two complementary strands were then annealed and labeled with [α-^{32}P]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 DTT) 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 0.5 X Tris-borate buffer, and the gels were then dried and examined by autoradiography.

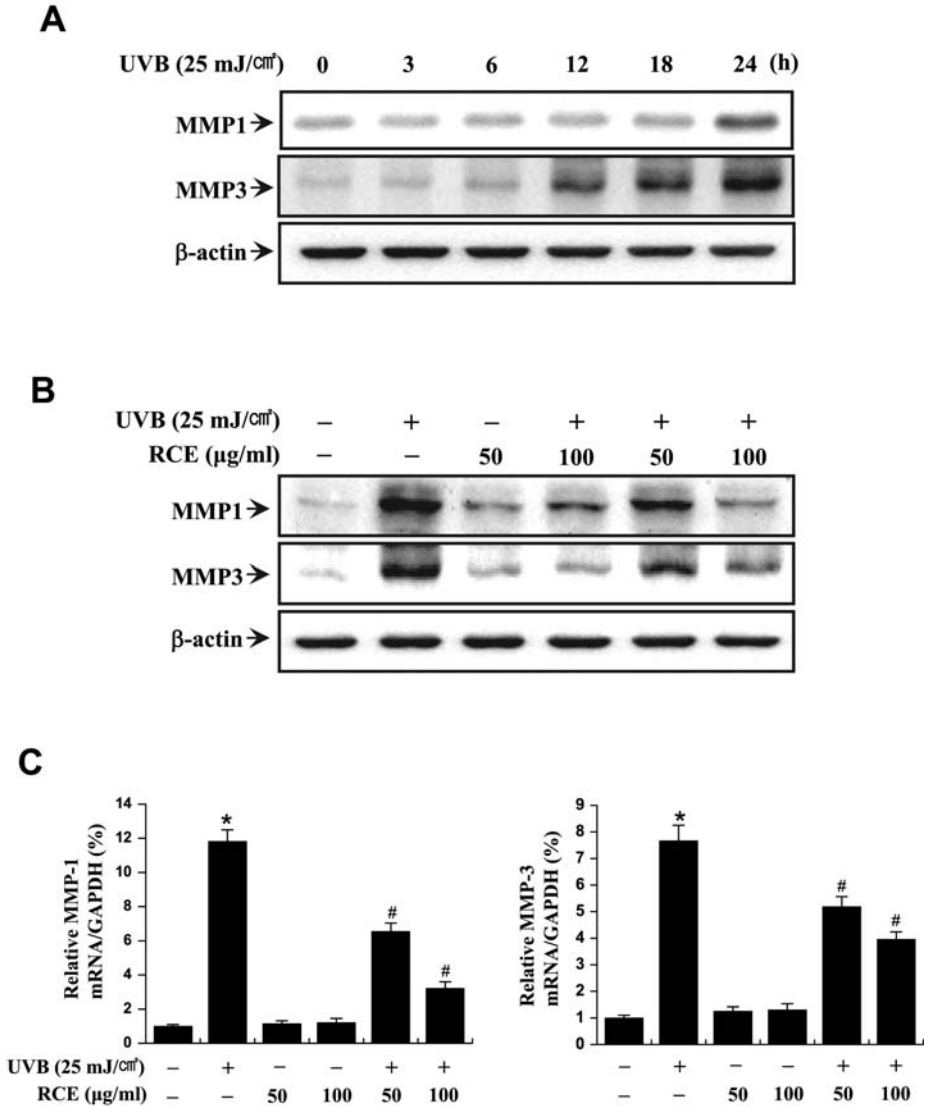

Figure 2. Effect of RCE on UVB-induced MMP-1 and -3 expressions in HDF. HDF in monolayer was incubated with stimulated UVB (25 mJ/cm^2) in a time-dependent manner (A). To investigate the effect of RCE, cells were stimulated with UVB (25 mJ/cm^2) and the indicated concentrations of RCE for 24 h (B). The cell lysates were analyzed by Western blotting with anti-MMP-1 and -3. The blot was reprobed with anti-ß-actin to confirm equal loading. HDF was pretreated with cordycepin for 1 h and stimulated by IL-1ß for 24 h. Total cellular RNA was analyzed by real-time PCR for MMP-1 and -3 (C). Each value represents the mean ±SEM of three independent experiments. *p<0.01 vs. untreated control; $^\#$p<0.01 vs. UVB.

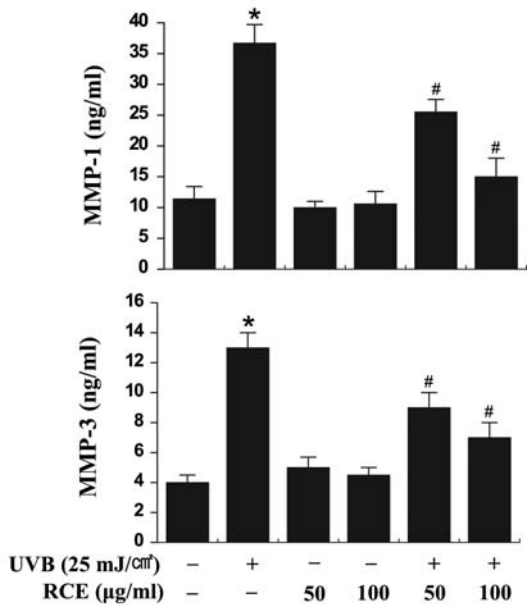

Figure 3. Inhibition of UVB-induced MMP-1 and -3 secretions from HDF by RCE. Cells were stimulated with UVB (25 mJ/cm^2) and the indicated concentrations of RCE for 24 h. Level of MMP-1 and -3 in culture media was measured using a commercially available ELISA kit as described in Materials and methods. Each value represents the mean ±SEM of three independent experiments. *p<0.01 vs. untreated control; #p<0.01 vs. UVB.

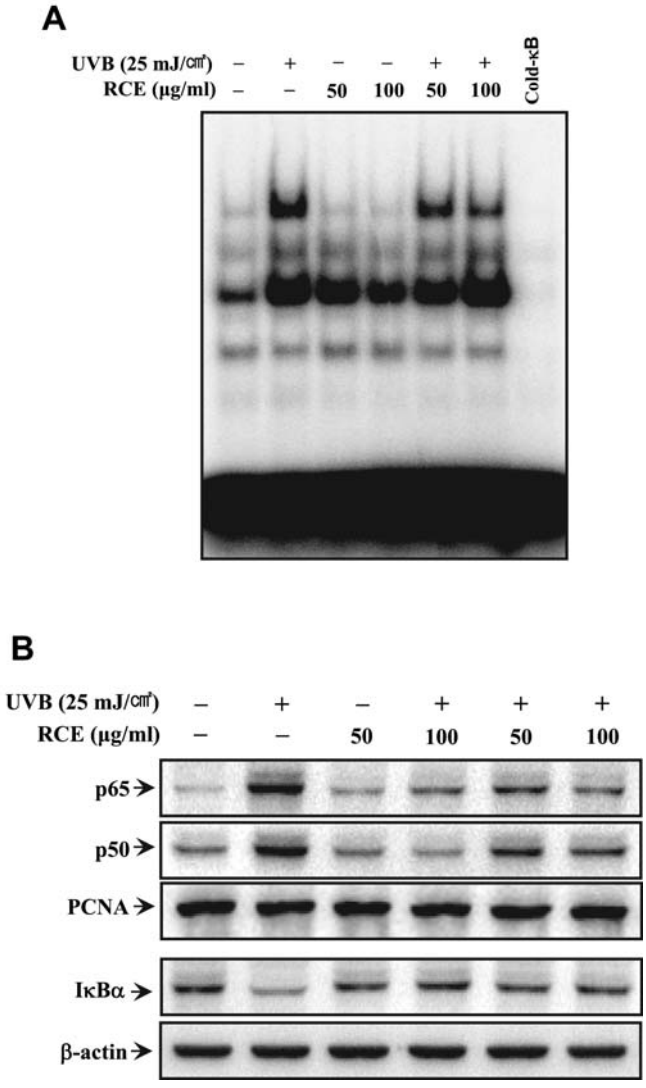

Figure 4. Suppression of IL-1ß-induced DNA binding of NF-κB, translocation of p65 and p50 to the nucleus, and IκBα degradation by RCE. Cells were stimulated with UVB (25 mJ/cm^2) and the indicated concentrations of RCE. Following 3 h of incubation, DNA binding of NF-κB was analyzed by electrophoretic mobility shift analysis (A), and the translocation of p65 and p50 to the nucleus and IκBα degradation in the cytoplasm (B) were determined by Western blotting.

Specific binding was controlled by competition with a 50-fold excess of cold κB oligonucleotide.

Statistical analysis. Statistical analysis of the data was performed using ANOVA and Duncan's test. Differences of $p<0.05$ were considered statistically significant.

Results

Effect of RCE on cell viability of HDF. Cytotoxicity of RCE on HDF has not yet been reported. Therefore, we designed to avoid interference of cytotoxicity from reagent. In this study, the effect of RCE on cellular toxicity of HDF was analyzed using the MTT assay. MTT assays were performed to determine optimal non-toxic concentrations at 24 h after incubation with RCE and no change in morphology. As shown in Fig. 1, treatment of HDF with indicated concentrations of RCE for 24 h resulted in no significant effect in cell viability.

Effect of RCE on UVB-induced MMP-1 and -3 expressions in HDF. To investigate UVB-induced MMP-1 and -3 expression, we performed Western blot analysis and real-time PCR in HDF. Western blot analysis revealed that the irradiation of HDF with UVB (25 mJ/cm^2) increased the levels of MMP-1 and -3 in a time-dependent manner (Fig. 2A). Treatment with RCE (50 and 100 μg/ml) completely blocked the up-regulation of MMP-1 and -3 induced by UVB irradiation (Fig. 2B). Real-time PCR revealed that UVB irradiation increased the level of MMP-1 and -3 in HDF and RCE blocked UVB-induced up-regulation of MMP-1 and -3 in a dose-dependent manner (Fig. 2C). As shown, RCE itself had no effect on either MMP-1 or -3 in HDF. These results indicate that RCE is a potent inhibitor of UVB-induced MMP-1 and -3 expression in HDF.

Effect of RCE on UVB-induced secretion of MMP-1 and -3. MMP secretion, which is the hallmark of skin aging, is activated by UVB, and 23 members of the MMP family have been identified in humans to date. Of these, MMP-1 and -3 are particularly important to skin aging because they are responsible for the degradation of collagenous extracellular matrix, and because levels of MMP-1 and -3 are significantly higher in aged skin. We used ELISA to investigate the effect of RCE on UVB-induced MMP secretion. Irradiation of HDF with UVB (25 mJ/cm^2) resulted in an increase in the secretion of MMP-1 and -3, respectively (Fig. 3). However, RCE (50 or 100 μg/ml) significantly diminished the UVB-induced MMP-1 and -3 secretions, respectively (Fig. 3).

ve effect of RCE on UVB-induced NF-κB activation. as implicated in the transcriptional regulation of UVB-induced MMP expression (14). Therefore, we studied the effect of RCE on UVB-stimulated translocation of NF-κB from the cytoplasmic compartment to the nucleus and on DNA binding in HDF. UVB-irradiated HDF showed increased binding activity of an NF-κB consensus sequence (Fig. 4A), as well as increased p65 and p50 subunit levels in their nuclei (Fig. 4B) when compared to unstimulated cells. Additionally, UVB-induced NF-κB activation was significantly suppressed by pretreatment with RCE, which suggests that RCE inhibits MMP-1 and -3 expression through the inhibition of NF-κB activation. The specificity of the DNA-protein interactions for NF-κB was demonstrated by performing competition assays using a 50-fold excess of unlabeled oligonucleotide (Fig. 4A, lane 7).

From above, we found that RCE blocked UVB-induced NF-κB activation. Under basal condition, the cytoplasmic protein IκB directly binds to p65 and p50 subunits and represses their nuclear translocation (15-17). Therefore, we determined the alteration of IκBα levels of the cytoplasmic fraction in this study. UVB-irradiated HDF showed a decreased level of IκBα protein in the cytoplasm when compared to a similar fraction in unstimulated cells, however, the increased IκBα degradation as a result of UVB stimulation was significantly suppressed by treatment with RCE (Fig. 4B).

Discussion

In this study, we demonstrated that RCE inhibits UVB-induced expression of MMP-1 and -3 in cultured HDF. Our results also showed that RCE blocked UVB-induced activation of NF-κB, which has an important role in MMP-1 and -3 expressions.

Skin aging is attributed to extrinsic (photoaging) and intrinsic (chronological) aging. Photoaging concerns premature skin aging caused by repeated sun exposure (18-20). UV irradiation of cultured HDF *in vitro* or human skin *in vivo* induces the expression of MMPs which play important roles in the degradation of extracellular matrix components during skin aging (7,21,22). Varani *et al* reported that with increasing age, MMP levels rise and collagen synthesis declines for sun-protected human skin *in vivo* (23). Recently, it was suggested that excessive matrix degradation by UV-induced MMPs secreted by various cells (e.g., keratinocytes, fibroblasts, and inflammatory cells) contributes substantially to the connective tissue damage that occurs during photoaging (20,24,25). The following mechanism of photoaging was proposed. Initially, AP-1 and NF-κB are activated by UV light, and AP-1 and NF-κB driven MMPs such as MMP-1 and -3 are induced (24-26). These MMPs then degrade collagen, which results in a collagen deficiency in photodamaged skin and eventually causes skin wrinkling (27).

In this study, we observed an increase in the activation of NF-κB in the UVB-irradiated HDF, and the ability of RCE to protect against the UVB-induced MMPs expressions, suggesting that RCE inhibits UVB-induced expression of MMPs by suppressing the NF-κB pathway in HDF. It is well established that a nuclear transcription factor, NF-κB, is activated upon UV irradiation (10,28,29). In previous studies, it was also reported that UVB-mediated skin photoaging is prevented by suppression of NF-κB activation (15,30,31). Therefore, inhibition of the NF-κB activation pathway is important to avoid UVB-mediated skin damage. In fact, NF-κB is known to increase MMP-1 in the dermis (14,32,33). NF-κB is a crucial factor for the immunoinflammatory responses and is also implicated in various skin diseases including allergic dermatitis, psoriasis vulgaris, and skin cancer (34). Hence, although NF-κB is involved in maintaining the skin homeostasis (35,36), excessive activation is pathogenic. NF-κB is an inducible dimeric transcription factor that belongs to the Rel/NF-κB family of transcription factors. NF-κB consists of two major polypeptides, p65 and p50 (37). NF-κB is initially located in the cytoplasm in an inactive form complexed with IκB, an inhibitory factor of NF-κB. Various inducers such as IL-1, TNF-α and UV cause dissociation of this complex, presumably by phosphorylation of IκB, resulting in NF-κB being released from the complex. NF-κB then translocates to the nucleus, where it interacts with specific DNA recognition sites to mediate gene transcription

In conclusion, the development of MMP inhibitors is considered a promising strategy for skin cancer therapy and photoaging. In recent years, the development of compounds with MMP inhibition activities from natural plants received a great deal of attention. This study demonstrates the inhibitory effect of RCE on the MMP expression via an mRNA assay. Also, our results demonstrate that RCE is a potent inhibitor of UVB-induced MMP expression and strongly blocks the ability of the NF-κB signaling pathway in HDF. The dose of RCE that inhibits UVB-induced MMP expression are below the toxicity limit. Therefore, we suggest that RCE should be viewed as a potential therapeutic candidate for preventing and treating skin photoaging.

Acknowledgements

This Study was supported by the Technology Development Program for Agriculture and Forestry, Ministry for Agriculture, Forestry and Fisheries, Republic of Korea.

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