Effects of Macelignan Isolated from Myristica fragrans (Nutmeg) on Expression of Matrix Metalloproteinase-1 and Type I Procollagen in UVB-Irradiated Human Skin Fibroblasts

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Exposure to ultraviolet (UV) light causes premature skin aging that is associated with upregulated matrix metalloproteinases (MMPs) and decreased collagen synthesis. Macelignan, a natural lignan compound isolated from Myristica fragrans HOUTT. (nutmeg), has been reported to possess antioxidant and anti-inflammatory activities. This study assessed the effects of macelignan on photoaging and investigated its mechanisms of action in UV-irradiated human skin fibroblasts (Hs68) by reverse transcription-polymerase chain reaction, Western blot analysis, 2'-7'-dichlorofluorescein diacetate assay, and enzyme-linked immunosorbent assay. Our results show that macelignan attenuated UV-induced MMP-1 expression by suppressing phosphorylation of mitogen-activated protein kinases (MAPKs) induced by reactive oxygen species. Macelignan also increased type I procollagen expression and secretion through transforming growth factor β (TGF-β)/Smad signaling. These findings indicate that macelignan regulates the expression of MMP-1 and type I procollagen in UV-irradiated human skin fibroblasts by modulating MAPK and TGF-β/Smad signaling, suggesting its potential as an efficacious anti-photodamage agent.

Key words macelignan; matrix metalloproteinase-1; type I procollagen; mitogen-activated protein kinase; UV irradiation; reactive oxygen species

Skin aging, which causes wrinkling, sagging and roughness, can be divided into two distinct processes: intrinsic aging and photoaging. Photodamage and premature skin aging is caused by ultraviolet (UV) irradiation, which increases the synthesis of matrix metalloproteinases (MMPs). MMP upregulation in skin fibroblasts accelerates the degradation of dermal collagen, which confers tensile strength. Skin aging is characterized by reductions in type I collagen, the primary component of the extracellular matrix (ECM), which provides structural support to the skin dermis. Therefore, natural compounds that decrease the MMP production and increase procollagen synthesis may have potential for the prevention and treatment of photoaging. Most are secreted as inactive proenzymes (proMMPs) that are activated primarily by proteolytic cascades, generally catalyzed by neutral proteases. MMP-1 is primarily responsible for degradation of ECM.

UV irradiation also leads to the generation of reactive oxygen species (ROS), direct or indirect DNA damage, and inflammatory response and damage to ECM integrity. UV-induced ROS generation stimulates cell surface cytokines, growth factor receptors, and mitogen-activated protein kinases (MAPKs), such as p38 kinase, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK), which in turn regulate activator protein-1 (AP-1). Increased AP-1 activity downregulates type I procollagen and upregulates MMP-1. The transcriptional activity of AP-1, a heterodimer composed of c-Jun and c-Fos, is dependent on the degree of c-Jun phosphorylation and c-Fos expression. UV irradiation also alters transforming growth factor β (TGF-β)/Smad signaling, which modulates ECM metabolism and tissue genesis through type I collagen production. The actions of TGF-β are enhanced by Smad3 and antagonized by Smad7.

Myristica fragrans HOUTT., commonly known as nutmeg, has traditionally been used as a spice and for medicinal purposes in several Asian countries. Macelignan (Fig. 1), a natural lignan compound isolated from nutmeg, has been reported to possess antioxidant, antiinflammatory, antibacterial, antifungal, hepatoprotective, and skin whitening activities. In addition, macelignan attenuates UVB-induced inflammation and photoaging in human keratinocytes by modulating MMP-9 and cyclooxygenase-2 (COX-2) expression. However, it is not known whether macelignan exerts photoprotective effects in human skin dermal fibroblasts. Therefore, this study was undertaken to evaluate the effects of macelignan on the expression of MMP-1 and type I procollagen in UVB-irradiated human skin fibroblasts and to determine its underlying mechanisms.

MATERIALS AND METHODS

Macelignan Macelignan (IUPAC name: (8R,8′S)-7-(3,4-methylenedioxyphenyl)-7′-(4-hydroxy-3-methoxyphenyl)-8,8′-dimethybutane) (Fig. 1) was isolated from Myristica fragrans HOUTT. (nutmeg) as previously described.

Cell Culture and UVB Irradiation Hs68 and CCD-986sk...
human skin fibroblasts were purchased from American Type Culture Collection (Manassas, VA, U.S.A.). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone Laboratories, Inc., Logan, UT, U.S.A.) supplemented with penicillin (120 units/mL), streptomycin (75 µg/mL), and 10% fetal bovine serum (HyClone Laboratories, Inc.) in an atmosphere of 5% CO2 at 37°C. The cells seeded in 6-well plates (2.0×10^5 cells/well) were pretreated with various concentrations of macelignan. After 24 h incubation, the culture medium was replaced with 100 µL MTI medium (0.5 mg/mL) and incubated for an additional 4 h. After washing the cells, the insoluble formaldehyde products were dissolved in 200 µL dimethyl sulfoxide. Absorbance at 550 nm was determined by spectrophotometry using a VersaMax tunable microplate reader (Molecular Devices Inc., Sunnyvale, CA, U.S.A.).

**Cell Viability** Cell viability was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, U.S.A.) colorimetric assay. Hs68 cells were cultured in 24-well plates (1×10^5 cells/well) for 6 h. The cells were then exposed to UVB (20 mJ/cm²) with a 312 nm UVB light source (VL-6.LM; Vilber Lourmat, U.K.) and visualized with the LAS 3000 Bio Imaging Analyser. Western blot analysis was then performed as previously described.20) The effects of macelignan on Hs68 cell viability were evaluated with the MTT assay. Hs68 cells were pretreated with macelignan (1–10 µM) for 24 h, washed with PBS, and stained with 40 µM fluorescein diacetate (FDCA; Sigma-Aldrich) for 30 min. The cells were then irradiated with UVB (20 mJ/cm²) and analyzed by flow cytometry (FACStar; Becton-Dickinson, San Jose, CA, U.S.A.).

**Enzyme-Linked Immunosorbent Assay (ELISA)** Hs68 cells were cultured in a 24-well plate (1×10^5 cells/well) and pretreated with macelignan (1–10 µM) for 24 h. The cells were then washed with PBS and irradiated with UVB (20 mJ/cm²) through a thin layer of PBS. After UVB irradiation, Hs68 cells were incubated with serum-free DMEM containing macelignan (1–10 µM). Cell culture medium was collected after 24 h, and type I procollagen production was quantified using a procollagen type I C-peptide enzyme immunoassay kit (MK101; Takara, Shiga, Japan).

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)** Total RNA was isolated from the cell pellet with Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.) and quantified by spectrophotometry at 260 nm. The cDNA was synthesized in a 20 µL reaction containing 1 µg total RNA, oligo (dT), and Reverse Transcription Premix (ELPIS-Biotech, Daejeon, Korea). PCR amplification of the cDNA products (5 µL) was performed with PCR premix (ELPIS-Biotech, Daejeon, Korea) and the following primer pairs (Bioneer, Daejeon, Korea): MMP-1 forward 5’-ATT CTA CTG ATA TCG GGG CTT TGA-3’, MMP-1 reverse 5’-ATG TCC TGG GAT GGC CAC ATC GG-3’ (366 bp); Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward 5’-TGA CCT TGG CCA GGG GTG CT-3’, GAPDH reverse 5’-CCA CCC GCA CAT CCG TC-3’ (517 bp). Before PCR amplification, primers were denatured at 94°C for 5 min. Amplification consisted of 22 cycles: denaturation at 94°C for 30 s, annealing at 56°C for 1 min, and extension at 72°C for 1 min, followed by a final 5 min extension at 72°C. PCR was performed in a Gene Amp PCR System 2700 (Applied Biosystems, Foster City, CA, U.S.A.). PCR products were separated by 1.5% agarose gel electrophoresis and visualized with ethidium bromide. GAPDH was used as an internal control.

**Measurement of ROS Production** Hs68 cells were pretreated with macelignan (1–10 µM) for 24 h, washed with PBS, and stained with 40 µM 2’7’-dichlorofluorescent diacetate (DCFH-DA; Sigma-Aldrich) for 30 min. The cells were then irradiated with UVB (20 mJ/cm²) and analyzed by flow cytometry (FACStar; Becton-Dickinson, San Jose, CA, U.S.A.).

**Statistical Analysis** All data are presented as mean± standard deviation (S.D.). Group results were compared by one-way analysis of variance, followed by the Scheffé test (SPSS 12.0); p<0.05 and p<0.01 were considered statistically significant.

**RESULTS**

**Cell Viability** The effect of macelignan on Hs68 cell viability was evaluated with the MTT assay. Hs68 cells were irradiated with UVB (20 mJ/cm²) and incubated with macelignan (0.5–20 µM) for 48 h. Cell viability was not affected by macelignan concentrations lower than 10 µM (data not shown).

**MMP-1 and Type 1 Procollagen Secretion** To evaluate the effects of macelignan on MMP-1 secretion in UVB-induced Hs68 cells, MMP-1 levels in the culture media were determined at 48 h by Western blot analysis. The results show that macelignan reduced MMP-1 secretion in a dose-dependent manner. Macelignan inhibited UV-induced MMP-1 expression by 10% at 1 µM, 31% at 5 µM, and 44% at 10 µM compared with the UV-irradiated control (Fig. 2A). Macelignan also inhibited MMP-1 expression of another human skin fibroblast cell line, CCD-986sk (data not shown). Type I procollagen protein levels in the culture media were assessed by ELISA. Treatment with macelignan increased type I procollagen protein secretion by 7% at 5 µM and 24% at 10 µM (Fig. 2B).

**MMP-1 and Type 1 Procollagen mRNA Levels** The effect of macelignan on MMP-1 and type I procollagen mRNA levels was evaluated by RT-PCR. Macelignan treatment reduced UVB-induced MMP-1 mRNA levels by 18% at 1 µM, 19% at 5 µM, and 36% at 10 µM compared with the UV-irradiated control (Fig. 3A). In addition, macelignan increased type
I procollagen mRNA levels by 56% at 5 µM and 105% at 10 µM (Fig. 3). These results indicate that macelignan inhibits UVB-induced ROS production.

**MAPK Signaling Pathway** MAPK signaling plays an important role in regulating MMP expression. To investigate the molecular mechanisms by which macelignan reduces MMP-1 levels, phosphorylation of ERK, JNK, and p38 was assessed. The results show that UVB-induced phosphorylation of ERK, JNK, and p38 was lower in macelignan-treated cells compared with the UVB-irradiated control (Fig. 5A). The results suggest that macelignan reduces MMP-1 expression by inhibiting the ERK, JNK, and p38 pathways. MAPK activation leads to the expression and phosphorylation of c-Jun and c-Fos, which form the AP-1 heterodimer, thereby upregulating MMP-1 transcription. Therefore, levels of c-Fos, c-Jun, and phosphorylated c-Jun were determined after UVB irradiation and macelignan treatment by Western blot analysis. Macelignan suppressed UVB-induced c-Jun phosphorylation; however, c-Jun and c-Fos levels were not significantly altered by macelignan (Fig. 5B).

**Smad3 and 7 Expression** TGF-β exerts its effects on cellular function by binding to the TGF receptor complex in cell-surface. Receptor-ligand binding triggers the Smad3 activation, which in turn regulates the expression of TGF-β target genes. Smad3 activation is suppressed by the endogenous negative regulator Smad7. To determine whether macelignan increases type I procollagen synthesis by blocking the effects of UVB irradiation on the TGF-β/Smad pathway, Smad3 and Smad7 protein levels in UVB-irradiated Hs68 cells were determined by Western blot analysis. Protein levels of Smad3 were increased by 80% at 1 µM, 132% at 5 µM, and 141% at 10 µM, whereas Smad7 levels were reduced by 46.9% at 1 µM in macelignan-treated cells compared with the UV-irradiated control, suggesting that macelignan downregulates Smad7 expression and upregulates Smad3 expression (Fig. 6).

**DISCUSSION**

UV irradiation induces MMP expression and reduces type I procollagen synthesis in the dermal ECM, leading to the loss of resiliency and increased laxity observed in photoaged skin. UV irradiation-induced ROS damage skin by altering gene expression and protein structure and function. In this study, the antiphotoaging action of macelignan and its underlying mechanisms were evaluated using UVB-irradiated human skin fibroblasts.

MMPs play a pivotal role in the pathophysiological mechanisms of photoaging. Specifically, UV irradiation alters the connective tissue of the skin by upregulating the expression of MMPs, which degrade collagen and other ECM proteins. Activated MMP-1, which is a member of the collagenase subfamily of MMPs, initiates collagen breakdown by cleaving type I and type III collagen, which are further degraded by MMP-2 and -9. Collagens confer strength and resiliency to skin; therefore, their disarrangement with photoaging causes the skin to appear aged. In the present study, macelignan markedly attenuated MMP-1 expression in a dose-dependent manner, and at 5 and 10 µM, restored type I procollagen protein and mRNA levels (Figs. 2, 3).
leads to the upregulation of MMP-1 and degradation of collagen in dermis and plays a critical role in MAPK signaling.\(^{21}\)

In this study, macelignan (1–10 \(\mu\)M) significantly reduced intracellular ROS production (Fig. 4). These results indicate that macelignan inhibits UVB-induced alterations in MMP-1 and type I procollagen levels, in part, by suppressing ROS generation.

MAPK signaling plays an important role in regulating MMP gene expression, cell motility, cell proliferation, and cell survival. Three distinct groups of MAPKs (JNK, ERK, and p38) have been identified in mammalian cells.\(^{25}\) A previous study reported that oxidative stress due to ROS accumulation initiates the MAPK signaling cascade by phosphorylation of MAPK proteins.\(^{25}\) In this study, UVB-induced phosphorylation of ERK, JNK, and p38 was lower in macelignan-treated cells, suggesting that macelignan attenuates UVB-induced MMP-1 expression by inhibiting MAPK signaling (Fig. 5A). MMP-1 gene expression is regulated by c-Jun and c-Fos, components of the AP-1 complex. Activated JNK phosphorylates c-Jun, enhancing its transcriptional activity.\(^{7,28}\) Macelignan inhibited UV-induced phosphorylation of c-Jun but did not affect c-Fos expression (Fig. 5B). Taken together, these results indicate that macelignan reduces UVB-induced c-Jun phosphorylation by inhibiting phosphorylation of JNK.

The individual polypeptide chains of type I and type III collagen are synthesized in dermal fibroblasts as procollagen.\(^{29}\) TGF-\(\beta\) regulates type I procollagen synthesis and downregulates the expression of the proteolytic enzymes, such as collagenase. TGF-\(\beta\) binds to TGF-\(\beta\) receptor complex, thereby regulating cellular functions. Receptor-ligand binding triggers the activation of Smad2 and Smad3, which induce the expression of TGF-\(\beta\) target genes. UV irradiation impairs TGF-\(\beta\)/Smad signaling, decreasing type I procollagen synthesis, leading to collagen loss in the dermis. Smad7 antagonizes TGF-\(\beta\) signaling by repressing Smad2 and Smad3. UV irradiation increases Smad7 mRNA and protein levels, which impairs TGF-\(\beta\)/Smad signaling.\(^{6,7}\) Smad3 transmits TGF-\(\beta\) signaling cascade from the cell-surface receptor to the procollagen gene promoter in human dermal fibroblasts.\(^{30,31}\) In this study macelignan increased Smad3 protein levels and decreased Smad7 protein levels (Fig. 6). These data show that macelignan restores impaired TGF-\(\beta\)/Smad signaling by regulating the expression of Smad3 and Smad7 (Fig. 6).

Natural compounds that decrease MMP production and increase procollagen synthesis may be able to attenuate skin photoaging. Our findings demonstrate that macelignan...
Fig. 5. Effects of Macelignan on MAPK Signaling

Hs68 cells were pretreated with different concentrations of macelignan for 24 h. After UV irradiation (20 mJ/cm²), the cells were cultured for an additional 1 or 2 h. (A) MAPK phosphorylation and (B) c-Jun and c-Fos phosphorylation/expression were evaluated by Western blot analysis. Each blot is representative of three independent experiments; α-tubulin was used as the loading control. Results are expressed as mean±S.D. (% control) of three independent experiments. *p<0.05, **p<0.01.
inhibits UVB-induced MMP-1 expression by suppressing oxidative stress and the associated intracellular signal pathways. Macelignan also appears to increase procollagen synthesis by stimulating TGF-β/Smad signaling. Our previous study demonstrated that macelignan attenuates UVB-induced photoaging and inflammation in epidermal keratinocytes by blocking MAPK and PI3K/Akt pathways, which regulate MMP-9 and COX-2 expression.17) Taken together, these findings show that macelignan exerts antiphotoaging effects in both fibroblasts and keratinocytes, indicating that macelignan shows promise for preventing and treating skin photoaging.

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REFERENCES


