**Meso-dihydroguaiaretic Acid from Machilus thunbergii Sieb et Zucc., and Its Effects on the Expression of Matrix Metalloproteinase-2, 9 Cause by Ultraviolet Irradiated Cultured Human Keratinocyte Cells (HaCaT)**

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Ethanol and aqueous extracts of Machilus thunbergii used traditionally for the treatments a wide variety of diseases were screened in vitro for the matrix metalloproteinase (MMP)-9 inhibitor actions. Meso-dihydroguaiaretic acid from the stems bark of Machilus thunbergii showed significant MMP-9 inhibition in human keratinocyte cells cause by ultraviolet irradiation. Here we investigated the effect of meso-dihydroguaiaretic acid, which was isolated from Machilus thunbergii, on UV-induced premature skin aging. We studied the effect of meso-dihydroguaiaretic acid on UV-induced MMP-9 expression in an immortalized human keratinocyte cell line, HaCaT, in vitro. Acute UV irradiation induced MMP-9 expression at both the mRNA and protein levels and meso-dihydroguaiaretic acid suppressed this UV-induced MMP-9 expression in a dose-dependent manner. Taken together, these results show that meso-dihydroguaiaretic acid can prevent the harmful effects of UV that lead to skin aging. Therefore, we suggest that meso-dihydroguaiaretic acid should be viewed as a potential therapeutic agent for preventing and/or treating premature skin aging.

Key words Machilus thunbergii; meso-dihydroguaiaretic acid; ultraviolet irradiation; matrix metalloproteinase (MMP)-9

Skin connective tissue contains several types of collagen, elastin, fibronectin, proteoglycan, and other extracellular matrix proteins, among which type I collagen is the most abundant. The balance between the synthesis and degradation of extracellular matrix components is important for maintaining a normal connective tissue structure. Moreover, it has been suggested that the most prominent features of photoaged skin are an impairment of collagen fiber and the excessive deposition of abnormal elastin-containing material in the upper and mid-dermis.11

The matrix metalloproteinases (MMPs) are a family of structurally related zinc-dependent endopeptidases, which are capable of degrading a wide variety of extracellular matrix components.2) MMPs are known to play important roles in tissue remodeling during developmental morphogenesis, angiogenesis, and tissue repair, and in tissue destruction during pathological processes, such as, arthritis, skin aging, tumor invasion, and metastasis. MMPs expression is low in unstimulated skin cells or normal skin tissues, but some MMPs are induced by various extracellular stimuli, e.g., ultraviolet or infrared radiation, growth factors, cytokines, and tumor promoters.12 Recent studies have shown that hairless mouse chronically exposed to ultraviolet B (UV-B) radiation show epidermal hyperplasia, skin wrinkles, and significant enhancement of gelatinase (i.e., MMP-2 and MMP-9) activities. In addition, they have shown that the inhibition of gelatinase activities by a specific MMP inhibitor, suppresses UVB-induced epidermal thickness enhancement and wrinkle formation.13 These results suggest that MMPs are directly involved in the skin photoaging process, and that the inhibition of the activities of MMPs (either directly by a specific inhibitor or indirectly by reducing their expression) may provide an effective therapeutic method of counteracting photoaging.

Many studies have shown that anti-oxidants including α-tocopherol, ascorbic acid, vitamins C and E, N-acetyl-cysteine (NAC), and genistein reduce UV-induced photoaging or carcinogenesis in human or hairless mouse skin.12 However, natural antioxidants like vitamin C and vitamin E are highly unstable and easily oxidized. In addition, other well known compounds such as NAC are only effective at unreasonably high working concentrations. Thus, there is a need for the identification and development of new antioxidants.

Machilus thunbergii Sieb et Zucc. (Lauraceae) is deciduous tree grown in the Southern areas of Korea.6) The resin from this species has been used in traditional medicine to treat edema, abdominal pain, and abdominal distension and was also used by Korea, China and Japan as both incense, and a folk medicine.7) Lignans,8)–11) alkaloids,12) flavonoids13,14) butanolides15) and essential oils16) have been reported as components from M. thunbergii, some of which have hepatoprotective activity as antioxidants,17) antibacterial activity,10) and inhibitory activity on nitric oxide synthesis in activated macrophages.15)

During the course of our search for new MMP’s inhibitor from traditional medicine materials, we found that a meso-dihydroguaiaretic acid of the stem bark of M. thunbergii showed activity. In this study, we investigated the effects of meso-dihydroguaiaretic acid on UV-induced photoaging in cultured human keratinocytes in vitro. Meso-dihydroguaiaretic acid was found to suppress UV-induced MMP-9 expression at both the mRNA and protein levels in cultured HaCaT cells. Taken together, these results demonstrate that meso-dihydroguaiaretic acid can potentially prevent or ameliorate UV-induced premature skin aging (photoaging), and suggest that meso-dihydroguaiaretic acid has potential use as an agent for preventing and/or treating premature skin aging.

**MATERIALS AND METHODS**

**Materials** The stem barks of Machilus thunbergii were collected in April 2004 at Ullung island, Korea and dried at room temperature. The botanical identification was made by Dr. Tae Jin Kim. A voucher specimen of this raw material has been deposited at the herbarium of the Seoul National University (SNU-04-52-01). Cell culture media, antibiotics, and Trizol reagent were purchased from Life Technologies, Inc. (Rockville, MD, U.S.A.). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT, U.S.A.). Meso-dihydroguaiaretic acid (Fig. 1) was prepared as described.17) N-Acetyl-cysteine (NAC) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

**Cell Culture and Compound Treatments** The immor-
talized human keratinocyte cell line, HaCaT, was cultured in Dulbecco’s modified Eagle’s media (DMEM) supplemented with glutamine (2 mM), penicillin (400 U/ml), streptomycin (50 mg/ml), and 10% FBS in a humidified 5% CO₂ atmosphere at 37 °C. For treatment, HaCaT cells were maintained on culture media without FBS for 48 h and then pretreated with meso-dihydroguaiaretic acid or NAC at the indicated concentrations for 1 h. After ultraviolet irradiation, they were then further incubated in fresh culture media without serum in the presence of meso-dihydroguaiaretic acid or NAC for 24 h.

Ultraviolet Irradiation  The UV light source was a F75/85W/UV21 fluorescent sun lamps, having an emission spectrum between 285—350 nm (peak at 310—315 nm) as previously described. A Kodacel filter (TA401/407; Kodak, Rochester, NY, U.S.A.) was mounted 2 cm in front of the UV tubes to remove wave lengths <290 nm (UV-C). Then the cells were exposed to UV (75 mJ/cm²) light.

RNA Analysis  Total RNA was prepared from cultured HaCaT cells using the Trizol method, as described by the manufacturer (Life Technologies, Inc., Rockville, MD, U.S.A.). Isolated RNA samples were electrophoresed in 1.0% agarose gels to assess the quality and quantity of RNA obtained. Extracted total RNA (1 μg) was reverse transcribed using a first strand cDNA reverse transcription-polymerase chain reaction (RT-PCR) synthesis kit (MBI Fermentas, Vilnius, Lithuania). Semiquantitative PCR was performed using primers for human GAPDH (forward, 5’-ATT GTT GCC ATC AAT GAC CC-3’; reverse, 5’-AGT AGA GGC AGG GAT GAT GTG-3’), for human MMP-2 (forward, 5’-GGC CAA GTG GTC GTG-3’; reverse, 5’-GAG GCC CCA TAG AGC TCC-3’) and for human MMP-9 (forward, 5’-CCC GGA CCA AGG ATA CAG-3’; reverse, 5’-CAG TAC CGA GAG AAA GCC-3’). The PCR conditions used were: initial denaturation (5 min at 94°C), 21 amplification cycles for GAPDH or 28 amplification cycles for MMP-2, 9 (both for 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C), followed by a final extension (10 min at 72°C). Reaction products were electrophoresed in 2.0% agarose gels and visualized with ethidium bromide. The signal strengths were quantified using a densitometric program (TINA; Raytest Isotop).
topenmebergerate, Straußenhardt, Germany). No PCR products were obtained for control reactions in which reverse transcriptase was omitted. After normalizing versus GAPDH intensities, percentage increases or decreases were determined. Each experiment was repeated at least five times.

**Zymography** Quantitative analysis of gelatinase activity by zymography was used to measure the relative amounts of MMP-2, 9 present. To determine the amount of MMP-2, 9 secreted into culture media by human keratinocytes, equal aliquots of conditioned culture media containing equal cell densities were fractionated using precast zymogram gels containing gelatin, according to the manufacturer’s protocol (NOVEX, San Diego, CA, U.S.A.). After electrophoresis, the gels were incubated in renaturing buffer (50 mM Tris–HCl, pH 7.4, 2% (v/v) Triton X-100) for 30 min at room temperature and in developing buffer (50 mM Tris–HCl, pH 8.0, 2.5 mM CaCl₂, 0.02% (w/v) sodium azide) for 72 h at 37°C. Proteolytic bands were visualized by staining with 0.5% (w/v) Coomassie Brilliant Blue solution. Signal strengths were quantified using a densitometric program (TINA; Raytest Isotopenmeberate, Straußenhardt, Germany).

**Nitroblue Tetrazolium Reduction Assay** The intracellular superoxide generation was measured by using the conversion of nitroblue tetrazolium to formazan. Nitroblue tetrazolium was added to the medium of cells to a final concentration of 1 mg/ml. After the drug treatment, cells were lysed and formazan was dissolved with 2 M KOH and 1.4 volume of dimethyl sulfoxide. The absorbance was read spectrophotometrically at 654 nm.

**Statistical Analysis** Data are presented as the mean± S.E.M. and were analyzed by analysis of variance and by unpaired t-test. A value of p<0.05 was considered to indicate statistical significance.

**RESULTS AND DISCUSSION**

Previous reports have shown that the activation of gelatinases including MMP-2 and MMP-9 play important roles in UVB-induced skin aging in hairless mice and that various antioxidants affect the expressions of MMPs induced by UV irradiation. Since meso-dihydroguaiaretic acid (Fig. 1) has strong antioxidant activity, we first tested whether it affected UV-induced MMP-9 expression in HaCaT cells, an immortalized human keratinocyte cell line. N-Acetyl-cysteine (NAC) was used as a control, because it has been widely used as an antioxidant agent in a variety of experiments. We pretreated HaCaT cells with various concentrations of meso-dihydroguaiaretic acid or NAC for 1 h, and then irradiated them with UV at 75 mJ/cm². Fresh culture media were added and the cells were further incubated for 24 h. Cells and culture media were harvested for mRNA and protein level analysis, respectively. As shown in Fig. 2, treatment of cells with meso-dihydroguaiaretic acid or NAC suppressed UV-induced MMP-9 expression at both the mRNA (Fig. 2A) and protein (Fig. 2B) levels. However, meso-dihydroguaiaretic acid was active at much lower concentrations (0.1—1 μM) than NAC (5—20 μM). These results suggest that meso-dihydroguaiaretic acid is more effective than NAC at inhibiting UV-induced MMP-9 expression in cultured HaCaT cells in vitro. As shown in Fig. 3, to investigate the effect of meso-dihydroguaiaretic acid on MMP-2 expression in ultraviolet irradiated human keratinocyte cell line. However, MMP-2 mRNA and protein levels were unaffected. This study describes the beneficial effects of a lignan compound, meso-dihydroguaiaretic acid, on the detrimental effects of UV in vitro. Meso-dihydroguaiaretic acid significantly inhibited UV-induced MMP-9 expression in cultured human keratinocytes in vitro. Gelatinases including MMP-2 and MMP-9 are known to degrade type IV and VII collagens, which are the principal components of the epidermal basement membrane. Moreover, recent studies have shown that UV-induced gelatinase activities play an important role in the processes of UV-induced skin damage, including skin thickening and wrinkle formation. Reactive oxygen species play a role in the ultraviolet irradiation-induced expression of MMPs through redox regulatory transcription factors. In accordance with previous other reports, our data also demonstrated that ultraviolet B irradiation increased reactive oxygen species production (Fig. 4). More importantly, meso-dihydroguaiaretic acid profoundly suppressed the reactive oxygen species generation induced by ultraviolet B irradiation (Fig. 4).

Therefore, it is likely that the suppression of UV-induced
MMP-9 expression by *meso*-dihydroguaiaretic acid prevents, to some extent, UV-induced skin damage in human keratinocyte cells. Further work is required to elucidate the protective mode of action of *meso*-dihydroguaiaretic acid.

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REFERENCES