Matrix Metalloproteinase-1 Inhibitory Activities of *Morinda citrifolia* Seed Extract and Its Constituents in UVA-Irradiated Human Dermal Fibroblasts

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The objective of this study was to examine whether a 50% ethanolic extract (MCS-ext) of the seeds of *Morinda citrifolia* (noni) and its constituents have matrix metalloproteinase-1 (MMP-1) inhibitory activity in UVA-irradiated normal human dermal fibroblasts (NHDFs). The MCS-ext (10 µg/mL) inhibited MMP-1 secretion from UVA-irradiated NHDFs, without cytotoxic effects, at 48 h after UV exposure. The ethyl acetate-soluble fraction of MCS-ext was the most potent inhibitor of MMP-1 secretion. Among the constituents of the fraction, a lignan, 3,3'-bisdemethylpinoresinol (**1**), inhibited the MMP-1 secretion at a concentration of 0.3 µM without cytotoxic effects. Furthermore, **1** (0.3 µM) reduced the level of intracellular MMP-1 expression. Other constituents, namely americanin A (**2**), quercetin (**3**) and ursolic acid (**4**), were inactive. To elucidate inhibition mechanisms of MMP-1 expression and secretion, the effect of **1** on mitogen-activated protein kinases (MAPKs) phosphorylation was examined. Western blot analysis revealed that **1** (0.3 µM) reduced the phosphorylations of p38 and c-Jun-N-terminal kinase (JNK). These results suggested that **1** suppresses intracellular MMP-1 expression, and consequent secretion from UVA-irradiated NHDFs, by down-regulation of MAPKs phosphorylation.

Key words *Morinda citrifolia*; noni seed; matrix metalloproteinase-1 inhibitor; photoaging; 3,3'-bisdemethylpinoresinol

Normal human dermis primarily consists of an extracellular matrix of connective tissue, of which the most abundant protein is collagen. Chronic UV exposure denatures collagen and elastic fibers in the dermis and induces wrinkles, thereby promoting photoaging.¹⁾ UV induces the production of matrix metalloproteinases (MMPs) by activating intracellular signal transcription pathways, including mitogen-activated protein kinase (MAPK) p38 and c-Jun-N-terminal kinase (JNK).²⁾ Activation of the phosphorylation of MAPKs may lead to an over expression of c-Jun and c-Fos. This results in induction of activator protein-1 (AP-1), which, in turn, up-regulates expression of MMPs.³⁾ MMPs are a family of structurally related matrix degrading enzymes that are associated with various destructive processes, including inflammation, tumor invasion and skin aging.¹⁾ MMPs are subdivided in different types at least over 20 members.⁴⁾ Among them, MMP-1, secreted from human skin fibroblasts, is mainly responsible for the degradation of dermal collagen in the photoaging process of human skin.⁵⁾ Neutrophils play an important role during the process of photoaging, which are set in motion by UV irradiation. The neutrophils infiltrate into the skin and release several active enzymes, such as MMPs and human leukocyte elastase (HLE).⁶⁾ HLE cleaves the helix structure of type I collagen and then degrades elastic fiber in human skin.⁷⁾ HLE also directly activates MMP-1 from human dermal fibroblasts.⁸⁾ Thus, it is expected that MMP-1 inhibitors and/or HLE inhibitors may be useful for the prevention of photoaging of human skin.

Morinda citrifolia L. (Rubiaceae), commonly known as “noni” in Hawaii and Tahiti, is a tropical tree. The fruits, roots, bark and leaves of noni have been used throughout Polynesia as a folk medicine for the treatment of many diseases.⁹⁾ Recently, the noni fruit juice and tea made from noni

leaves have been introduced to the functional food market. Noni fruit contains a large number of seeds throughout its flesh. During the production of noni fruit juice, these seeds are removed and discarded. Consequently, we have investigated the utility of noni seed and previously reported that a 50% ethanolic extract from noni seeds (MCS-ext) had more potent *in vitro* HLE inhibitory activity than the extracts of noni leaves or fruit flesh.¹⁰⁾ MCS-ext also exhibited tyrosinase inhibitory and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities. Activity-guided fractionation of MCS-ext led to the isolation of 3,3'-bisdemethylpinoresinol (**1**), americanin A (**2**) and quercetin (**3**) as active constituents with both tyrosinase inhibitory and DPPH radical scavenging activities. Ursolic acid (**4**) was isolated as a triterpenoid with potent HLE inhibitory activity. Compounds **2** and **3** also showed superoxide dismutase like activity.¹⁰⁾ If MCS-ext is proven to be MMP-1 inhibitor, an extract with HLE inhibitory activity may be an ideal ingredient for anti-photoaging cosmetics. The objective of this study was to determine if MCS-ext and its constituents have MMP-1 inhibitory activity in UVA-irradiated normal human dermal fibroblasts (NHDFs).

MATERIALS AND METHODS

Plant Materials and Reagents Fruits of *M. citrifolia* were collected in French Polynesia during 2004–2006. The sample was identified by Tropical Resources, Inc. (Provo, UT, U.S.A.). The fruits were separated into flesh and seeds by hand. The seeds were air-dried. Voucher specimens of dried seeds (Noni Seed: 004-239A-0014) are deposited at Kinki University. The preparation of 50% ethanolic extract of dried seed (MCS-ext, yield 5%) and the isolation of lignans (**1**, **2**) have been described in our previous paper.¹⁰⁾ Quercetin

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(3) and ursolic acid (4) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Epigallocatechin-3-*O*-gallate (EGCG) was purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Other chemical and biochemical reagents were of reagent grade and were purchased from Wako Pure Chemical Industries, Ltd. and/or Nacalai Tesque, Inc. (Kyoto, Japan) unless otherwise noted.

Fractionation of MCS-ext A suspension of MCS-ext (30 g) in water (120 mL) was extracted with hexane (120 mL×2) followed by ethyl acetate (120 mL×10). Evaporation of solvent from each combined extract gave a hexane-soluble fraction (0.29 g; yield from MCS-ext: 1%) and an ethyl acetate-soluble fraction (5.4 g; 18%). The aqueous layer was evaporated and then lyophilized to give a water-soluble fraction (22 g; 73%).

Cell Culture Normal human dermal fibroblasts (NHDFs) obtained from neonatal foreskins was purchased from Kurabo Industries, Ltd. (Osaka, Japan) in Feb. 2010. NHDFs were precultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Nichirei Biosciences Inc., Tokyo, Japan) at 37°C in a humidified CO₂-controlled (5%) incubator. NHDFs from passages 3 to 6 were used for the experiments.

Assay of Cell Viability The cell viability was determined by a 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (WST-8) assay using a commercial kit (Tetra Color ONE, Seikagaku Corporation, Tokyo, Japan). Test samples were dissolved in dimethyl sulfoxide (DMSO) and then diluted with DMEM to an appropriate concentration. In the control group, 0.2% (v/v) DMSO/DMEM solution was used instead of sample solution. The final concentration of DMSO was 0.2% (v/v). Briefly, 1×10⁴ cells in the medium (100 μL) were placed on 96-well microplate. After 24 h of incubation, several concentrations of samples in the medium (100 μL) were added. After incubation for 48 h, the medium was replaced with a new medium (10% WST-8/DMEM, 100 μL). After incubation for 1 h, the resulting formazan was photometrically determined at 450 nm with a microplate reader. Cell viability was expressed as a percentage of the control cells.

UV Irradiation NHDFs were cultured until confluent on 6-well microplate. The cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and exposed to UVA irradiation (365 nm; FL20S•BLB/DMR, Toshiba Co., Tokyo, Japan) in PBS (1 mL). The total energy dose of UVA irradiation was 5 J/cm². Using the trypan blue (Life Technologies Japan Ltd., Tokyo, Japan) exclusion test, the UV dose did not affect the cellular viability (data not shown). After UVA exposure, the cells were washed twice with PBS and the test sample solution (2 mL) was immediately added to each well. In the control and the vehicle control groups, 0.2% (v/v) DMSO/DMEM solution was used instead of sample solution. In the vehicle control and test groups, NHDFs were exposed to UVA.

Western Blot Analysis for MMP-1 in Cultured Medium After irradiation, NHDFs were incubated at 37°C for various lengths of time in serum free medium on 6-well microplate. Aliquots of the cultured NHDFs medium were centrifuged at 12000×*g* for 15 min at 4°C. The supernatant was boiled for 4 min in sodium dodecyl sulfate (SDS)-containing sample buffer (Bio-Rad Laboratories, Hercules, CA, U.S.A.) containing 5% (v/v) β-mercaptoethanol and then chilled on ice. An equal volume of resulting supernatant

was separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories). The membranes were subsequently blocked overnight at 4°C with 5% (w/v) skim milk in Tris-buffered saline (TBS; 25 mM Tris, 137 mM NaCl, 2.68 mM KCl) containing 0.1% Tween 20 and then incubated with anti-MMP-1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.) at 1:400 dilution for 1 h at room temperature. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated anti-goat immunoglobulin G (IgG) secondary antibody (Santa Cruz Biotechnology Inc.) at 1:4000 dilution for 1 h at room temperature. The resulting proteins were detected by enhanced chemiluminescence Western blot detection reagents kit (ECL Plus, GE Healthcare U.K. Ltd., Little Chalfont, Buckinghamshire, U.K.), and visualized with the ChemiDoc XRS Plus System (Bio-Rad Laboratories Inc.). Relative band density was determined by Quantity One 1-D Analysis Software (Version. 4.6.7, 2008; Bio-Rad Laboratories Inc.).

Western Blot Analysis for MAPKs and Intracellular MMP-1 The cultured NHDFs were washed twice with pre-cooled PBS (pH 7.4) and then lysed with a cell lysis buffer (Cell Signaling Technology, Danvers, MA, U.S.A.) containing 1 mM phenylmethanesulfonyl fluoride. The lysates were centrifuged at 12000×*g* for 15 min at 4°C. The supernatant was subjected to Western blot analysis, as described above. Protein concentrations of samples were determined by DC protein assay reagent (Bio-Rad Laboratories Inc.) with bovine serum albumin (Bio-Rad Laboratories Inc.) as a standard. Equal amounts of samples were separated by 10% SDS-PAGE and transferred onto PVDF membranes. The membranes were subsequently blocked with 5% (w/v) skim milk in TBS containing 0.1% Tween 20 or Blocking One-P overnight at 4°C and then incubated with the indicated antibodies. The antibody against MMP-1 (Santa Cruz Biotechnology Inc.) was used at 1:400 dilution. The membranes were exposed to horseradish peroxidase-conjugated anti-goat IgG secondary antibody (Santa Cruz Biotechnology Inc.) at 1:4000 dilution. Antibodies against p38, phospho-p38, JNK, phospho-JNK and β-actin were purchased from Cell Signaling Technology and used at 1:1000 dilution. Subsequently, the membranes were exposed to the horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (GE Healthcare U.K. Ltd.) at 1:2000 dilution. The resulting proteins were detected by ECL Plus, and analyzed by ChemiDoc XRS Plus System, as described above.

Statistical Analysis Each experiment was performed in triplicate unless otherwise noted. Intergroup differences were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. GraphPad Prism for Windows, Ver. 5 (GraphPad Software Inc., 2007) was used for the statistical analyses.

RESULTS

Effect of MCS-ext on MMP-1 Secretion from UVA-Irradiated NHDFs MMP-1 protein in the culture medium was assessed by Western blot analysis. NHDFs were exposed to UVA at a dose of 5 J/cm². UVA irradiation time-dependently enhanced the secretion of MMP-1 from NHDFs in the vehicle control group at 9 to 48 h, as compared to the con-

Fig. 1. Effect of MCS-ext on MMP-1 Secretion from UVA-Irradiated NHDFs

Fibroblasts were exposed to UVA (5 J/cm^2) and then cultured in the serum free medium containing test samples for 9, 24 and 48 h. MMP-1 protein levels in the cultured medium at the indicated times were assessed by Western blot analysis using the antibody against human MMP-1. The blot is representative of three separate experiments and represents a single immunoblot.

Fig. 2. Effects of Hexane-, Ethyl Acetate- and Water-Soluble Fractions from MCS-ext and EGCG on MMP-1 Secretion from UVA-Irradiated NHDFs

Fibroblasts were exposed to UVA (5 J/cm^2) and then cultured in the serum free medium containing test samples for 48 h. MMP-1 protein levels in the cultured medium were assessed by Western blot analysis using the antibody against human MMP-1. The blot is representative of three separated experiments and represents a single immunoblot. EGCG (epigallocatechin-3-*O*-gallate) was used as a reference compound.

control group without UVA irradiation (Fig. 1). UVA-irradiated NHDFs were treated with MCS-ext ($10\text{ }\mu\text{g/mL}$) immediately after UVA exposure. MCS-ext obviously inhibited the secretion of MMP-1 from UVA-irradiated NHDFs at 9 to 48 h. MCS-ext had no cytotoxic effect at 3 to $30\text{ }\mu\text{g/mL}$ (cell viability: $98\pm 2\%$, $98\pm 6\%$, $99\pm 4\%$ at 3, 10, $30\text{ }\mu\text{g/mL}$, respectively). Thus, we assessed the level of MMP-1 secretion into the culture medium at 48 h when the inhibition of MMP-1 secretion was most distinctly detected.

Effects of Hexane-, Ethyl Acetate- and Water-Soluble Fractions Obtained from MCS-ext and EGCG on MMP-1 Secretion from UVA-Irradiated NHDFs Fractionation of MCS-ext by successive solvent extraction gave a hexane-, an ethyl acetate- and a water-soluble fraction. Effects of these fractions and EGCG, a well known inhibitor of MMP-1 secretion,¹¹⁾ on MMP-1 secretion from UVA-irradiated NHDFs were assessed. Results of Western blot analysis are shown in Fig. 2. EGCG showed potent inhibition of MMP-1 secretion at a concentration of $0.1\text{ }\mu\text{M}$. Although all three fractions ($3\text{ }\mu\text{g/mL}$) significantly inhibited the MMP-1 secretion at 48 h compared to the vehicle control group, the ethyl acetate-soluble fraction showed the most potent inhibition, but had no cytotoxic effect at 1 to $10\text{ }\mu\text{g/mL}$ (cell viability: $102\pm 11\%$, $98\pm 5\%$, $102\pm 5\%$ at 1, 3, $10\text{ }\mu\text{g/mL}$, respectively).

Effects of Constituents of MCS-ext and EGCG on MMP-1 Secretion from UVA-Irradiated NHDFs As reported in our previous paper,¹⁰⁾ 3,3'-bisdemethylpinoresinol (**1**, isolation yield from MCS-ext: 1.17%; isolation yield from dried seeds: 0.065%), americanin A (**2**, 0.51%; 0.024%), quercetin (**3**, 0.19%; 0.009%) and ursolic acid (**4**, 0.74%; 0.035%) have been isolated from the ethyl acetate-soluble fraction. The effects of these constituents on MMP-1 secretion were examined by Western blot analysis, and the relative intensity of each blot shown in Fig. 3Aa was measured. The serum free medium containing each constituent at a concentration of 0.03, 0.1 and $0.3\text{ }\mu\text{M}$ was added to UVA-irradiated NHDFs im-

mediately after UVA exposure, and the mixture was incubated for 48 h. As shown in Figs. 3Aa and Ab, **1** ($0.3\text{ }\mu\text{M}$) obviously reduced the levels of MMP-1 secretion, compared to the vehicle control group. On the other hand, **2** (Fig. 3Ba), **3** (Fig. 3Bb) and **4** (Fig. 3Bc) were inactive at $0.3\text{ }\mu\text{M}$. In the range of 0.03 to $0.3\text{ }\mu\text{M}$, **1**, **2**, **3** and **4** had no cytotoxic effect, as shown in Fig. 3C.

Effects of 3,3'-Bisdemethylpinoresinol (1**) on Intracellular MMP-1 Expression in UVA-Irradiated NHDFs** To elucidate the mechanism of MMP-1 inhibition, we examined the effect of **1** on intracellular MMP-1 expression in UVA-irradiated NHDFs, as well as MMP-1 secretion into the culture medium. The time-course of intracellular MMP-1 expression and MMP-1 secretion after UVA-irradiation is shown in Fig. 4. In the vehicle control group, UVA irradiation gradually enhanced the levels of intracellular MMP-1 expression during 24 to 48 h. The most distinct MMP-1 expression was detected at 48 h, as shown in Fig. 4A. Compound **1** ($0.3\text{ }\mu\text{M}$) obviously inhibited the increase of intracellular MMP-1 expression at 48 h. The levels of MMP-1 secretion into the cultured medium from UVA-irradiated NHDFs were increased at 48 h in the vehicle control group, whereas the treatment of the cells with **1** obviously inhibited MMP-1 secretion (Fig. 4B).

Effect of UVA Irradiation on the Levels of MAPKs Phosphorylation in NHDFs To further understand the mechanism of MMP-1 inhibition in NHDFs by **1**, the activation of MAPKs after exposure to UVA irradiation (5 J/cm^2) was preliminarily examined by Western blot analysis. The time-courses of JNK and p38 phosphorylation levels are shown in Fig. 5A. UVA irradiation resulted in a rapid and transient activation of JNK phosphorylation at 0.5 to 1.0 h after exposure. The p38 phosphorylation levels were remarkably enhanced in a rapid and transient manner at 0 to 1.0 h after exposure. The basal levels of JNK and p38 in NHDFs were not affected by UVA irradiation.

Fig. 3A. Effects of 3,3'-Bisdemethylpinoresinol (**1**) and EGCG on MMP-1 Secretion from UVA-Irradiated NHDFs

Fibroblasts were exposed to UVA (5J/cm²) and then cultured in the serum free medium containing test samples for 48h. MMP-1 protein levels in the cultured medium were assessed by Western blot analysis using the antibody against human MMP-1. (a) The blot is representative of three separated experiments and represents a single immunoblot. (b) All data are reported as mean±S.E. of three separate experiments. **p*<0.05 and ***p*<0.01: statistically significant vs. the value of vehicle control group.

Fig. 3B. Effects of Americanin A (**2**), Quercetin (**3**), and Ursolic Acid (**4**) and EGCG on MMP-1 Secretion from UVA-Irradiated NHDFs

Fibroblasts were exposed to UVA (5J/cm²) and then cultured in the serum free medium containing test samples for 48h. MMP-1 protein levels in the cultured medium were assessed by Western blot analysis using the antibody against human MMP-1. The blot is representative of three separate experiments and represents a single immunoblot.

Fig. 3C. Effects of 3,3'-Bisdemethylpinoresinol (**1**), Americanin A (**2**), Quercetin (**3**) and Ursolic Acid (**4**) on Cell Viability

Fibroblasts were incubated in serum free cultured medium in the presence of the indicated concentration of samples for 48h. Cell viability was determined by a WST-8 assay. All data was given as mean±S.E. of three separated experiments.

Effect of 3,3'-Bisdemethylpinoresinol (1**) on the Levels of MAPKs Phosphorylation in UVA-Irradiated NHDFs** Effect of **1** (0.1, 0.3μM) on JNK and p38 phosphorylation in UVA-irradiated NHDFs was examined. On the basis of above preliminary results (Fig. 5A), phosphorylation levels were assessed at 0.5h after UVA-irradiation. As shown in Fig. 5B, **1** (0.3μM) obviously inhibited the phosphorylation of both JNK and p38 at 0.5h after, whereas a lower concentration of **1** (0.1μM) produced no significant inhibition.

DISCUSSION

MMP-1 is a major collagenolytic enzyme responsible for collagen damage in UV-irradiated human skin.¹²⁾ It has been demonstrated that UV irradiation enhances MMP-1 expression in NHDFs¹³⁾ and increases MMP-1 secretion into culture medium.¹⁴⁾ In the past decade, a number of papers have reported the isolation of MMP-1 inhibitors from plant resources. Examples include aucubin from *Eucommia ulmoides* cortex,¹⁵⁾ tectoridin-4'-*O*-β-D-glucoside from aerial parts of *Viola hondoensis*,¹⁶⁾ sumafllavone and amentoflavone from whole plant of *Selaginella tamariscina*,¹⁷⁾ styraxjaponoside B and erythrodiol-3-acetate from *Styrax japonica* stem,^{18,19)} xanthorrhizol from *Curcuma xanthorrhiza* rhizome,¹⁴⁾ caffeic acid and chlorogenic acid from *Coffea arabica* leaf,²⁰⁾ panduratin A and 4-hydroxypanduratin A from *Kaempferia pandurata* rhizome,^{21,22)} *Punica granatum* extract,²³⁾ *Embolica officinalis* extract²⁴⁾ and *Sanguisorba officinalis* extract.²⁵⁾ Among these plants, a few plant such as *Coffea arabica* leaf extract²⁰⁾ is known to have HLE inhibitory activity. It has been demonstrated that MCS-ext exhibits HLE inhibitory activity.¹⁰⁾ HLE inhibitors with MMP-1 inhibitory activity are ideal ingredients for anti-photoaging cosmetics. Therefore, as part of our continuing investigation of noni seeds, we examined MCS-ext for MMP-1 inhibitory activity. UVB-irradiated NHDFs were used to assay MMP-1 inhibitory activity of almost all constituents of the plants cited above. Further, UVA-induced photo-damage of the skin is believed to be the result of effects on dermal fibroblasts by deeply penetrating UVA radiation, as opposed to UVB radiation which only penetrates the epidermis.¹³⁾ Therefore, we used UVA-irradiated NHDFs for the evaluation of MMP-1 inhibitory activity. UVA irradiation was performed at 5J/cm², according to a previously reported method²⁶⁾ in which the UV dose enhanced MMP-1 secretion without any significant effects on cell viability.

The MCS-ext significantly reduced the secretion of MMP-1 without any cytotoxic effects. Activity guided fractionation of MCS-ext was carried out to identify the active constituents of MCS-ext by successive solvent extractions. The ethyl acetate-soluble fraction exhibited the most potent activity. Therefore, we focused on the fraction from which two lignans (**1**, **2**), **3** and **4** were previously isolated as active constituents having potent DPPH radical scavenging, tyrosinase inhibitory and HLE inhibitory activities.¹⁰⁾ The MMP-1 inhibitory activities of **1**, **2**, **3** and **4** were assayed. The lignan (**1**) concentration-dependently displayed potent inhibitory activity without any significant effects on cell viability.

Compound **3** had no inhibitory activity at 0.3μM. But the effect of **3** on MMP-1 is controversial. Leu *et al.*²⁷⁾ reported that **3** (50μg/mL, 166μM) inhibited MMP-1 secretion from NHDFs at 24h. In addition, Lim and Kim²⁸⁾ reported that **3**

Fig. 4. (A) Effect of 3,3'-Bisdemethylpinoresinol (**1**) on the Intracellular MMP-1 Expression in UVA-Irradiated NHDFs, (B) Effect of 3,3'-Bisdemethylpinoresinol (**1**) on MMP-1 Secretion from UVA-Irradiated NHDFs

(A) Fibroblasts were exposed to UVA (5 J/cm^2) and then cultured in the serum free medium containing test samples for 4, 8, 24 and 48 h. Intracellular MMP-1 protein levels at the indicated times were assessed by Western blot analysis using the antibody against human MMP-1. The blot is representative of two separate experiments and represents a single immunoblot.

(B) Fibroblasts were exposed to UVA (5 J/cm^2) and then cultured in the serum free medium containing test samples for 24 to 48 h. MMP-1 protein levels in cultured medium were assessed by Western blot analysis using the antibody against human MMP-1. The blot is representative of two separate experiments and represents a single immunoblot.

Fig. 5. (A) Time Course of the Phosphorylation Levels of MAPKs in UVA-Irradiated NHDFs, (B) Effect of 3,3'-Bisdemethylpinoresinol (**1**) on the Levels of MAPKs Phosphorylation in UVA-Irradiated NHDFs

(A) Fibroblasts were exposed to UVA (5 J/cm^2) and then cultured in the serum free medium for the indicated time. Total cellular proteins were prepared for Western blot analysis of MAPKs and phospho-MAPKs proteins using the antibodies against phospho-form and total-form of JNK and p38. β -Actin was used as an internal control. The blot is representative of three separate experiments and represents a single immunoblot.

(B) Fibroblasts were exposed to UVA (5 J/cm^2) and then cultured in the serum free medium for 0.5 h. Total cellular proteins were prepared for Western blot analysis of MAPKs and phospho-MAPKs proteins using the antibodies against phospho-form and total-form of JNK and p38. β -Actin was used for internal control. The blot is representative of three separate experiments and represents a single immunoblot.

($25\mu\text{M}$) down-regulated MMP-1 expression and secretion in 12-*O*-tetradecanoylphorbol 13-acetate-treated NHDFs at 24 h, but showed no effect at a concentration of $2.5\mu\text{M}$. In contrast, Cho *et al.*²⁹ recently described that **3** ($20, 40\mu\text{M}$) enhanced MMP-1 expression in NHDFs at 24 h, but was inactive at a concentration of $10\mu\text{M}$. Considering the content of **3** in MCS-ext, we used a dose of **3** (0.03 to $0.3\mu\text{M}$) in the experiments. Our inactive result of **3** at a low concentration ($0.3\mu\text{M}$) was not contradictory to the reported data.^{28,29} However, there remained a discrepancy of the effect of **3** at high concentration (20 to $166\mu\text{M}$) on MMP-1 expression. Although we assumed that a part of the discrepancy might be due to the differences among several experimental conditions, further studies are required to prove the precise effect of **3** on MMP-1 in NHDFs.

Furthermore, **1** significantly inhibited the increase of intracellular MMP-1 expression, and decreased the level of MMP-1 secretion (Fig. 4). These results indicated that the inhibition of MMP-1 secretion by **1** was due to down-regulation of MMP-1

expression. Considering the content of **1** in MCS-ext, at least a portion of MMP-1 inhibitory activity of the extract may be attributed to **1**.

Since **1** is newly described as a MMP-1 inhibitor, we attempted to elucidate its MMP-1 suppression mechanism. MAPKs signaling plays an important role in various cellular process, including MMP-1 secretion.⁵ Klotz *et al.*³⁰ reported that UVA radiation in human skin fibroblasts induces a pattern of MAPKs activation consisting of rapid and transient induction of p38 and JNK activity but not extracellular signaling-regulated kinase. UVA irradiation (5 J/cm^2) to NHDFs resulted in a rapid and transient activation of the JNK phosphorylation. The level of p38 phosphorylation was remarkably enhanced in a more rapid and transient manner. These results (Fig. 5A) are in agreement with the reported data.³⁰ We examined the effect of **1** on phosphorylation levels of MAPKs. The results (Fig. 5B) suggest that **1** may decrease JNK and p38 phosphorylation and suppress c-Fos and c-Jun expression,

followed by inhibition of AP-1 and MMP-1 expression, as well as MMP-1 secretion.

In conclusion, MCS-ext significantly inhibited MMP-1 secretion from UVA-irradiated NHDFs. The lignan (**1**) was isolated from MCS-ext as an active constituent. Western blot analysis suggested that **1** inhibited the secretion of MMP-1 through reduction of JNK and p38 phosphorylation and intracellular MMP-1 expression. This is the first report of the MMP-1 inhibitory activity of noni. Since MCS-ext has both MMP-1 inhibitory and HLE inhibitory activities, the extract may be useful for the prevention of skin photoaging.

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