Anti-Skin Aging Effect of Syriacusins from *Hibiscus Syriacus* on Ultraviolet-Irradiated Human Dermal Fibroblast Cells

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Abstract — Photosensitized peroxidation of membrane lipids has been implicated in skin pathologies such as phototoxicity and premature aging. We have previously reported that syriacusin compounds isolated from *Hibiscus Syriacus* inhibited lipid peroxidation. Here, we investigated whether syriacusins could be effective inhibitor to skin aging using ultraviolet-irradiated human dermal fibroblast cells (HDFCs). Syriacusins A, B, and C inhibit the activity of human neutrophil elastase (HNE), a serine protease to degrade extracellular matrix (ECM) proteins including elastin, with IC50s of 8.0, 5.2, and 6.1 μM, respectively. No changes in cell viability were detected by syriacusins A and B in UV-B (10 mJ/cm²) irradiated HDFCs. Matrix metalloproteinase (MMP)-1 expression in HDFCs was increased by UV-B irradiation. MMP-1 expression in UV-B irradiated HDFCs was decreased by 10 μM and 20 μM syriacusin A to 50% and 20% of untreated control, respectively. Syriacusin B treated with 20 μM reduced MMP-1 expression in UV-B irradiated HDFCs to 60% of untreated control. Syriacusin A also inhibited MMP-2 expression accompanying the increase of type-I pro-collagen in UV-B irradiated HDFCs. These results demonstrate that syriacusin A could be a more effective compound to inhibit skin aging caused by UV irradiation. It suggests that syriacusins A and B might be developed as possible agents to treat or prevent skin aging.

Keywords: Syriacusin, *Hibiscus syriacus*, MMP-1, Pro-collagen, Human dermal fibroblast

INTRODUCTION

Skin aging is a complex biological process that affects skin function and appearance. Skin connective tissue contains several types of collagen, elastin, fibronectin, proteoglycan and other extracellular matrix (ECM) proteins, among which type-I collagen is the most abundant. (Kligman, 1969; Bernstein et al., 1994; Rittie and Fisher, 2002). Human skin aging resulting from ultraviolet (UV) irradiation is a cumulative process that occurs based on the degree of exposure to sunlight (Imokawa, 2008; Imokawa, 2009). Quantitative and qualitative changes in the dermal ECM proteins are associated with photo-damage and eventually skin aging (Imokawa, 2009).

Elastin is an important structural protein of ECM and the major component of elastic fibers that provides resilience and elasticity to many tissues including skin, lungs, ligaments and arterial walls (Wiedow et al., 1990; Tsukahara et al., 2006). The reduction of elastin in skin plays a role in the formation of wrinkles (Tsuij et al., 2001). Human neutrophil elastase (HNE) is a serine protease located primarily in the azurophil granules of polymorphonuclear leukocytes. HNE has a broad substrate specificity being able to degrade ECM proteins such as elastin, collagen, fibronectin, laminin, proteoglycan (Wiedow et al., 1990; Tsuij et al., 2001; Tsukahara et al., 2006) and the other connective tissue proteins such as cartilage tissues (Antonicelli et al., 2007). Biologically, elastase activity increases significantly with age and results in reduced skin elasticitic properties (Labat-Robert et al., 2000; Tsukahara et al., 2001; Tzaphlidou, 2004). Inhibition of elastase activity may protect skin aging (Kim et al., 2009; Xu et al., 2010).

The matrix metalloproteinases (MMPs) are a family of structurally related zinc-dependent endopeptidases that can degrade a wide variety of extracellular matrix compo-
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Fig. 1. Isolation procedure of syriacusins A, B, and C from Hibiscus syriacus.

MATERIALS AND METHODS

Plant materials

Hibiscus syriacus was collected at Yuseong, Chungnam Province, Korea, and identified by staff at the Korea Research Institute of Bioscience and Biotechnology (KRIIBB), Korea. A voucher specimen is deposited in the herbarium of KRIIBB.

Extraction and isolation of active constituents from Hibiscus syriacus

Active ingredients were extracted and isolated by the described previously method (Yoo et al., 1998). Briefly, the dried root bark and of H. syriacus (1.6 kg) were extracted twice with methanol for 2 days at room temperature and then the extracts were partitioned by n-hexane, chloroform, ethyl acetate and n-butanol in turn. Among them, chloroform layer was subjected to column chromatography over a silica gel eluting with n-hexane:EtOAc (10:1-1:1, v/v) gradient system. Fractions were collected and combined by monitoring with HNE inhibitory activity in combination with analytical TLC to yielded subfractions 1-1 and 1-2. Subfraction 1-1 was rechromatographed on silica gel column eluting with same solvents as described above, and then purified by Sephadex LH-20 with CHCl3-MeOH (1:1). Syriacusin A (35.5 mg) was finally purified from subfraction 1-1 by HPLC on a Senshu pak ODS column (20×250 mm) with 70% aq. MeOH at 8 ml min⁻¹. Subfraction 1-2 was further purified by column chromatography over silica gel eluting with CHCl3-MeOH (100:1 to 1:1) followed by
Sephadex LH-20 with methanol, to give syriacusin C (3 mg) and another active subfraction 1-2-2. Syriacusin B (3.5 mg) was purified from subfraction 1-2-2 by silica gel prep TLC developed with CHCl3-MeOH (10:1) (Fig.1, 2). For the bioassy test, compounds were dissolved in DMSO at 100 mM and stored at −20°C. Epigallocatechin gallate (EGCG) was used as a positive control.

**Instrumental analysis**

NMR spectra were recorded on Varian UNITY 300 spectrometer at 300 and 500 MHz for 1H, and 75 and 125 MHz for 13C in CDCl3 + CD3OD (1:1), CDCl3 and DMSO-d6 with TMS as an internal standard. Complete proton and carbon assignments were based in 1D (1H, 13C, DEPT) and 2D (1H-1H COSY, 1H-13C HMQC, 1H-13C HMBC) NMR experiments. Mass spectra (HREI-MS) were measured using a JMS-SX 102A (JEOL). HPLC was performed on a Senshu pak ODS column (20×250 mm, YMC, RP-18, Japan) using a H2O-MeOH system and by monitoring with a photodiode-array detector (Waters 515 pump, 2,996 photodiode array detector, USA). HNE (Calbiochem) inhibitory activity was measured according to the ELISA reader VersaMax (Molecular Devices, USA).

**Cell culture**

Human skin fibroblast cells (CRL-2076) were purchased from the American Type Culture Collection (ATCC). Cell were cultured in Dulbecco’s Eagle’s medium (DMEM: Gibco) supplemented with penicillin A (100 U/ml), streptomycin (100 U/ml), and 10% heat-inactivated fetal bovine serum (Gibco). Cells were maintained in a humidified incubator 5% CO2 atmosphere at 37°C

**MTT assay**

The tetrazolium dye colorimetric test was used to determine the viability of fibroblast cells (Denizot and Lang, 1986). For treatment, cells were maintained on culture media without FBS for 24 h and UV-B irradiation. Then, cells were treated with syriacusins at concentrations range from 0, 3, 10, and 30 μM. MTT solution was added after an incubation period of 48 h. Cells were incubated at 37°C for an additional 3 h. The supernatant was then removed, and 150 μl of dimethyl sulfoxide (DMSO) was added. Absorbance was measured on a microplate reader at 570 nm to obtain the percentage of viable cells as compared to control group.

**UV irradiation**

Human dermal fibroblast cells (HDFCs) were grown in 6-well culture plate (BD Falcon) and maintained in culture media without FBS overnight. The cells were rinsed twice with phosphate-buffered saline (PBS), and the cells were exposed to UV light under a thin layer of PBS (Gibco). Cells were immediately washed with PBS after irradiation. Then, cells were cultured for 48 h in the serum-free media with or without syriacusins. The same conditions without UV irradiation were used for the control group.

**Human neutrophile elastase (HNE) assay**

The HNE inhibitory activity of syriacusins A, B, and C were evaluated using a previously described procedure (Kim et al., 2009; Xu et al., 2010). Briefly, each well of 96-well plate containing 40 μl of substrate solution [1.4 mM N-methoxysuccinyl-alal-alapro-val-p-nitroanilide in 10 mM Tris-HCl buffer (pH 7.5)], 50 μl of test solution (stock solutions of the test compounds were dissolved in DMSO and diluted with Tris-HCl buffer to give the final sample concentrations), adding 10 μl of an enzyme solution (0.18 units HNE) were mixed and incubated for 1 h at 37°C in the dark. After the reaction was quenched by adding 100 μl of soybean trypsin inhibitor at a concentration of 0.2 mg/ml, and the absorbance was immediately measured at 405 nm using ELISA reader. HNE inhibitory activity was expressed as follows:
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Table I. Inhibitory effect of syriacusins A, B, and C on human neutrophile elastase (HNE) activity

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Inhibition ratio for HNE (%)</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 μM</td>
<td>3 μM</td>
</tr>
<tr>
<td>Syriacusin A</td>
<td>19.7 ± 1.6</td>
<td>25.7 ± 1.7</td>
</tr>
<tr>
<td>Syriacusin B</td>
<td>15.3 ± 1.8</td>
<td>30.1 ± 2.1</td>
</tr>
<tr>
<td>Syriacusin C</td>
<td>16.5 ± 2.2</td>
<td>22.9 ± 1.0</td>
</tr>
<tr>
<td>EGCGg</td>
<td>45.3 ± 1.6</td>
<td>63.0 ± 0.5</td>
</tr>
</tbody>
</table>

*Data are expressed as the means ± S.D. (n=3), and IC50 indicated the concentration (μM) at which the percentage inhibition of HNE activity was 50%. **EGCG, (-)-epigallocatechin-3-gallate** was used as a positive control.

Western blot analysis

Cells were lysed in ice-cold lysis buffer containing 0.5% Nonidet P-40 (v/v) in 20 mM Tris-HCl (pH 8.3); 150 mM NaCl; protease inhibitors (2 μg/ml aprotenin, pepstatin, and chymostatin; 1 μg/ml leupeptin and pepstatin; 1 mM phenylmethyl sulfonyl fluoride (PMSF); and 1 mM Na2VO3. Lysates were incubated for 30 minutes on ice before centrifugation at 12,000×g for 5 minutes at 4°C. Proteins in the supernatant were denatured by boiling for 5 minutes in sodium dodecyl sulfate (SDS) sample buffer. Proteins in equal volume of conditioned culture media or equal number of cells were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Amersham international). To block the non-specific interaction between, membranes were incubated with 5% skim milk in Tris-buffered saline with Tween 20 (TBST) (10 mM Tris-HCl, pH 7.6; 150 mM NaCl; 0.5% Tween 20) for 1 h. To determine the amounts of MMP-1 (42/46 kDa) and type-I pro-collagen (170-190 kDa) secreted into culture media, membranes were incubated with the monoconal anti-MMP-1 antibody (Calbiochem) and polyclonal anti-pro-collagen type-I antibody (Santa Cruz Biotechnology Inc.) diluted at 1:1,000 and 1:500, respectively. Bound antibodies were incubated with HRP-conjugated anti-mouse IgG and anti-goat IgG antibody as a secondary antibody diluted at 1:1,000 and 1:500 dilution, respectively. Molecules were detected with the use of enhanced ECL system (Amersham international). Signal strength of each molecule was quantified by using a densitometric program (TINA).

Zymography for MMP-2

MMP-2 was detected by zymography in 10% polyacrylamide gel containing 0.1% gelatin (invitrogen, USA) (Demeule et al., 2000). Insoluble debris was removed by centrifugation at 12,000 g for 5 min at 4°C and 15 μl of supernatant was mixed with Tris-glycine SDS sample buffer (2×) without reducing agent. Without boiling, 25 μl of sample was loaded on to 10% SDS-PAGE. After electrophoresis, SDS gel was incubated with 1× zymogram renaturing buffer (Invitrogen, USA) for 30 min at room temperature. Then, 1× zymogram developing buffer (Invitrogen, USA) was added to the gel. After developing for 30 min, a developing buffer was exchanged with fresh 1× zymogram developing buffer. After the gel was incubated at 37°C overnight, the gel was stained with 0.05% Commassie Brilliant Blue solution and destained with destaining solution containing 10% acetic acid, 40% methanol until protein bands were clearly visible in a blue background.

RESULTS

Inhibitory effect of syriacusins on human neutrophile elastase (HNE) activity

The chloroform soluble fraction of methanol extract from the root bark of *Hibiscus syriacus* had been bio-assayed for the isolation of active constituents (Fig. 1). Those are three naphthalene compounds, syriacusins A, B, and C (Fig. 2).

Given that lipid peroxidation was inhibited by syriacusins A, B, and C (Yoo et al., 1998) and photosensitized peroxidation of membrane lipids has been implicated in skin aging (Girotti and Kriska, 2004), we examined the effect of syriacusins on HNE activity in human dermal fibroblast cells (HDFCs). Syriacusins were used at 4 different concentrations, 1, 3, 10, and 30 μM. As shown in Table I, syriacusins A, B and C inhibit HNE activity with IC50s of 8.0, 5.2, and 6.1 μM, respectively. Their effect were comparable to the positive control, (-)-epigallocatechin-3-gallate (EGCG) with IC50 of 1.1 μM. It suggests that syriacusins could be a novel anti-skin aging agent to inhibit the degradation of elastin.

Cell viability had not been changed by syriacuisin A and B

To investigate whether the effects of syriacusins A, B and C on the UVB-induced MMP-1 expression and MMP-2
expression in cultured human dermal fibroblast cells (HDFCs), cells were irradiated with 0, 10, 20, and 30 mJ/cm² of UV-B. Cell viability was more than 90% up to 10 mJ/cm² as compared to UV-B untreated control (Fig. 3A). When HDFCs were exposed to 10 mJ/cm² and immediately further incubated for 48 h in the presence of syriacusins A, B, and C with the concentrations of 3, 10 and 30 μM, no changes were detected in cell viability as compared to syriacusins-untreated control (Fig. 3B). In the meanwhile, cell viability was significantly decreased by the incubation with syriacuasin C at 10 μM (data not shown). It demonstrates that syriacusins A and B could be safe compounds to treat UV-exposed skin.

**Syriacuasin A and B inhibit the expression of MMP-1 and MMP-2**

Given that HNE degrade ECM proteins such as elastin and collagen (Wiedow et al., 1990; Tsuji et al., 2001; Tsukahara et al., 2006) and MMP-1 is the major enzyme responsible for collagen 1 digestion (Dong et al., 2008), we investigated the effect of syriacusins A and B on the expression of MMP-1 protein levels in HDFCs. Cells were irradiation with UBV (10 mJ/cm²) and immediately incubated with 1, 2, 5, 10, and 20 μM syriacusins A or B for 48 h. As shown in Fig. 4A, syriacuasin A inhibited UV-B-induced MMP-1 expression in a dose-dependent manner compared to the UV-B-irradiated control. MMP-1 expression in

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**Fig. 3.** Syriacusins A and B did not change cell viability exposed to UV-B irradiation. (A) Human dermal fibroblast cells (HDFCs) were irradiated with various energy of UV-B prior to incubation for 48 h. Cells viability of HDFCs was measured by MTT assay. Data represent mean ± SED. *p < 0.05, **p < 0.01, cell viability in UV-B irradiated-treated group was significantly different from control. (B) HDFCs were exposed to UV-B (10 mJ/cm²) in the presence or absence of various concentrations of syriacusins A and B. Cells viability of HDFCs was measured by MTT assay.

**Fig. 4.** UV-B-induced matrix metalloproteinase (MMP)-1 expression was inhibited by syriacusins A and B in human dermal fibroblast cells (HDFCs). (A) and (B) HDFCs were exposed to UV-B (10 mJ/cm²) in the presence or absence of various concentrations of syriacusins A and B, respectively. MMP-1 expression was detected by Western blot analysis as described in materials and methods.
HDFCs to UV-B (10 mJ/cm²) enhanced MMP-2 expression. As shown in Fig. 5, the exposure of HDFCs to UV-B irradiation significantly decreased expression of MMP-1 (Fig. 4B). Syriacusin B treated with 20 μM reduced MMP-1 expression in UV-B irradiated HDFCs to 60% of untreated control. It demonstrates that syriacusin A could be a more effective compound to inhibit the degradation of collagen caused by UV irradiation.

Pro-collagen expression was increased by syriacusin A

To confirm the inhibitory effect of syriacusin A on MMP-1, we examined the effect of the production of type-I procollagen in UV-B-irradiated HDFCs. As shown in Fig. 5, the exposure of HDFCs to UV-B (10 mJ/cm²) suppressed the level of type-I pro-collagen in the culture medium. However, the reduced type-I pro-collagen synthesis by UV-B irradiation was significantly attenuated by the incubation with syriacusin A at 20 μM.

Given that MMP-2 activities were increased by a chronic exposure to UV-B in hairless mice (Inomata et al., 2003; Suganuma et al., 2010) and the inhibition of gelatinase activities suppresses UV-B-induced wrinkle formation (Inomata et al., 2003; Suganuma et al., 2010), we measured the changes of MMP-2 activity caused by UV-B irradiation in HDFCs. As shown in Fig. 5, the exposure of HDFCs to UV-B (10 mJ/cm²) enhanced MMP-2 expression. When HDFCs were incubated with syriacusin A at 20 μM immediately after UV-B irradiation, the increased MMP-2 expression was significantly attenuated to control level. It suggests that syriacucins A and B might be developed as possible agents to treat or prevent skin aging.

DISCUSSION

Regarding the environmental damage to skin, the most common physical injury is caused by UV irradiation. Photaged skin is biochemically characterized by predominance of abnormal elastic fibers in the dermis and by a dramatic decrease in distinct interstitial collagens. Elastin is an important component of elastic fibers and also involved in inhibiting or repairing wrinkle formation (Labat-Robert et al., 2000), although collagen is a major factor in the skin. Elastase is a metalloproteinase which acts on degradation of elastin (Labat-Robert et al., 2000; Tsukahara et al., 2001; Tzaphlidou, 2004). It is known that elastase activity is increased by UV-B radiation (Kim et al., 2009; Xu et al., 2010). MMP-mediated collagen damage is a major contributor to the phenotype of photoaged human skin (Varani et al., 2001). Hence, development of MMP inhibitor is considered to be a promising strategy for skin prevention and/or treatment of UV-induced skin damage (Inomata et al., 2003; Suganuma et al., 2010). We have examined the anti-wrinkle effects of various plant extracts including syriacucins A, B and C isolated from Hibiscus syriacus by screening the inhibitory effect on human neutrophil elastase (HNE). We also investigate whether syriacucins A, B and C are effective on the inhibition of UVB-induced MMP-1 expression and the production of type-I procollagen expression in cultured HDFCs. Data showed that syriacusin A inhibited UV-induced MMP-1 expression and enzymatic activity of MMP-2 and increased type-1 procollagen synthesis in UV-B irradiated HDFCs. However, a little inhibition to MMP-1 expression was shown by syriacusin B and a poor cell viability was detected by syriacusin C. It suggests that syriacusin A could be a more effective compound to inhibit skin aging caused by UV irradiation than syriacusin B or C.

UV irradiation is known to provoke oxidative stress through the generation of reactive oxygen species (ROS) in cells (Lee et al., 1999; Tsukahara et al., 2006; Imokawa, 2008; Imokawa, 2009). ROS generation could result in the subsequent activation of complex signaling pathways, followed by the damage on DNA in skin cell (Dong et al., 2008; Imokawa, 2008). Earlier investigations indicated that the MAP kinase-mediated signal transduction plays an important role in the regulating a variety of cellular functions, including MMP-1 expression (Reunanen et al., 2002; Di Girolamo et al., 2003; Cortez et al., 2007), type-I collagen (Amemiya et al., 1999; Touyz et al., 2001) and elastin (Choi et al., 2009) synthesis. Our result revealed that syriacucin A had no inhibitory effect on the intracellular ROS levels (data not shown). It implicates that the inhibitory ef-
fect of syriacusin A on MMP-1 is not mediated by its anti-
oxidant effect but may be resulted from the modulation of another intracellular signal transduction molecules. It is re-
quired to define the effects of syriacusin A on the cell signal pathways in the further study.

Collectively, syriacusins A, B and C were isolated from the Hibiscus syriacus. Among them, syriacusin A reduced the expression of MMP-1/2 and induced the expression of type-I procollagen at the protein level in UV-irradiated cultured HDFCs. It suggests that syriacusin A might be developed as a possible agent to treat or prevent skin aging.

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