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Inhibitory Effects of *Alaria fistulosa* Uronans on Reactive Oxygen Species Generation and Matrix Metalloproteinase-1 Expression

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Abstract This study aimed to evaluate the inhibitory effects of water-soluble uronans (the low-molecular-weight analogues of alginic acids) isolated from *Alaria fistulosa* on ROS generation and MMP-1 expression *in vitro* using an immortalized human keratinocyte cell line. HaCaT cells were pretreated with *A. fistulosa* uronans (0.1, 1 and 10 μg/ml, respectively) prior to UVB irradiation and incubated for 24 h. ROS production was determined via the DCFH-DA method. MMP-1 mRNA and protein were determined by RT-PCR and Western blot analysis, respectively. The effect of *A. fistulosa* uronans on MMP-1 promoter activity was measured by luminometer. Pretreatment with 10 μg/ml uronans significantly inhibited UVB-induced ROS generation and MMP-1 expression relative to UVB irradiation alone ($p<0.05$). Uronans significantly reduced MMP-1 mRNA expression and inhibited UVB-induced MMP-1 promoter activity at concentrations of 1 and 10 μg/ml relative to UVB irradiation alone ($p<0.05$). Therefore, the results suggest that *A. fistulosa* uronans may be a potential therapeutic agent for the prevention and treatment of skin photoaging.

Keywords Uronan, Reactive oxygen species, Matrix metalloproeinase-1, Photoaging

Introduction

Long-term effects of repeated exposure to ultraviolet (UV) light have been associated with degenerative diseases, which commonly involve the degradation of the extracellular matrix and aberrant matrix metalloproteinase-1 (MMP-1) expression. Additionally, UVB irradiation has been shown to generate reactive oxygen species (ROS) in the cells and skin, which induces MMP synthesis, thus inducing photoaging of the skin [1]. In a variety of cell lines, MMP-1 expression is tightly regulated via a network of signals involving ROS [2]. ROS generation performs a critical function in the mitogen-activated protein kinase (MAPK)-mediated signal transduction triggered by UV [3]. Additionally, ROS scavengers have been shown to inhibit UV-induced activator protein-1 (AP-1) activation and MMP-1 expression [4]. It was recently reported that UV rapidly and significantly increases H_2O_2 levels in human skin *in vivo*, thus suggesting that an early increase in ROS may participate in the triggering of the MAPK cascade and that topical treatment with antioxidants, e.g. genistein and N-acetyl cysteine, may interrupt the activation of MAPK pathways and thereby inhibit UV-induced MMP expression in human skin *in vivo* [5].

The MMPs are a family of zinc-dependent endoproteinases that carry out pivotal roles in the dynamic remodeling of the extracellular matrix (ECM). The MMPs are responsible for the degradation or synthesis inhibition of collagen

extracellular matrix in connective tissues [6]. MMP-1 is the most abundant MMP and is secreted by a variety of cells, including keratinocytes, fibroblasts, and inflammatory cells, and contributes substantially to the connective tissue damage inherent to photoaging [7]. By potentially preventing imbalances of the synthesis/degradation of major fibrillar components of the ECM during the photoaging process, inhibitors of UV-induced MMP-1 may be a useful tool for maintaining ECM homeostasis [8]. Hence, the development of MMP-1 inhibitors is regarded as a promising strategy for skin cancer therapy and photoaging. Many studies concerning natural compounds with inhibitory effects on MMP-1 have been conducted thus far. For example, some flavonoid compounds, such as naringenin, apigenin, wogonin, kaempferol, and quercetin have already been shown to regulate MMP-1 [9].

Brown seaweeds are known to generate different polysaccharides: namely fucoidans, laminarans, and alginic acid [10]. These polysaccharides are of significant interest because of their specific biological activities [11]. The biological activities of polysaccharides depend on their fine structures and molecular weight. Presently, information regarding complex studies of water-soluble polysaccharides from brown algae has been rather scarce. Water-soluble polysaccharides are the waste products of the processing of brown algae, though they are of specific interest because of their possible application in medicine, agriculture, fish culture microbiology, etc.

Alaria fistulosa is a brown algal genus of the order Laminariales, family *Alariaceae*. It has been detected in the northwest Pacific on north Hokkaido, Kamtschatka, the Aleutian Islands, and Alaska. *A. fistulosa* contained almost exclusively water-soluble natural polyuronans (the low-molecular-weight analogues of alginic acids), with a small amount of fucoidan and a negligible quantity of laminaran (less than 1%) [12]. Relatively little data is currently available regarding the biological activities of the polyuronans contained in *A. fistulosa*. Therefore, the aim of this study was to evaluate the inhibitory effects of water-soluble uronans isolated from*A. fistulosa* on UVB-induced ROS generation and MMP-1 expression in human keratinocyte cells (HaCaT).

Materials and Methods

Cell culture - The immortalized human keratinocyte HaCaT cell line was obtained from Cell Line Service (Eppelheim, Baden-Wurttemberg, Germany). The cells were plated in 100 mm tissue culture dishes and grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (all obtained from GIBCO, Grand Island, NY, USA).*A. fistulosa* uronans were dissolved in distilled water. For treatment, the cells were maintained in culture media with 0.5% FBS-supplemented DMEM, followed by 24 h of treatment with *A. fistulosa* uronans. The cells were then rinsed twice in phosphate-buffered saline (PBS), and all UVB irradiation exposures were carried out under a thin layer of PBS (GIBCO, Grand Island, NY, USA). Immediately after irradiation, the cells were incubated in serum-free fresh culture media containing *A. fistulosa* uronans.

Polysaccharide extraction and separation - *A. fistulosa* samples were collected from Rashua Island, Sea of Okhotsk, in August 1999. The isolation and separation of water-soluble polysaccharides were conducted via a modified version of previously-described methods [12]. Fresh or deep-frozen seaweed (5 kg) was initially and successively treated with ethanol, acetone, and chloroform. Samples of the defatted, dried, and powdered algal fronds (500 g) were extracted with 0.1 M HCl (5 l) for 5 h at 60℃. The extracts were filtered and applied on the hydrophobic sorbent "Polychrome-1". Extracts were eluted from the hydrophobic sorbent "Polychrome-1" with water, followed by 15% aqueous ethanol [13]. Fractions eluted by water (fucoidans and uronans), were concentrated, centrifuged and precipitated with 60% ethanol. The precipitates obtained were washed in ethanol and acetone and dried under vacuum. A fraction of laminarans, eluted with 15% ethanol, was concentrated and precipitated with ethanol (1:4 v/v). The mixture of acidic polysaccharides (fucoidans and uronans) (1 g) was dissolved in water (50 ml) and acidified with acetic acid (30 ml). The obtained uronans gel was centrifuged (10,000 rpm, 10 min), washed twice in 40% acetic acid, redissolved in 1 M NaOH (50 ml) to pH 6.0 and dialyzed. The solution obtained was subjected to ultrafiltration on a 1000 NMWL Millipore membrane (Sigma, St. Louis, MO, USA), concentrated and precipitated with 2 volumes of ethanol. The precipitates were washed with ethanol and acetone, and dried under vacuum. The supernatant (which contained fucoidans), obtained after the precipitation of

uronans with acetic acid, was neutralized by NaOH (40%) to pH 6.5, dialyzed and precipitated with ethanol as described above.

Molecular mass determination - Molecular mass determination was carried out via gel-permeation FPLS on a column (1.5 × 30 cm) with Superdex 75 HR 10/30 (Amersham Pharmacia Biotech AB, Piscataway, NJ, USA). Elution was conducted with 0.1 M sodium phosphate buffer, ph 7.4 and 0.15 M NaCl, at a flow rate of 0.4 ml/h. Dextrans (10, 20, 40, and 80 kDa) were used as standards. Neutral carbohydrates were quantified via the phenol-sulfuric acid method [14].

^{13}C-nuclear magnetic resonance (NMR) - Spectra of ^{13}C-NMR for solutions of substances in D_2O were obtained on a Bruker Avance DPX-500 NMR spectrometer (Bruker, Berlin, Germany) with a working frequency of 75.5 MHz at 60℃.

Ultraviolet irradiation - The UV light source used was a Philips TL 20W/12RS fluorescent sun lamp (Amsterdam, Holland) with an emission spectrum of 285-350 nm (peak at 310-315 nm). The cells were then exposed to a 15 mJ/cm^2 dose of UVB light.

Assay for ROS production - ROS production was evaluated herein via the 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) method [1]. DCFH-DA which had entered the cell was cleaved to form DCFH. Trapped DCFH was oxidized by oxygen free radicals to yield fluorescent DCF. Cells treated with *A. fistulosa* uronans were incubated for 30 min with 20 μM of DCF-DA. Cells were harvested at the indicated time points after UV irradiation. Immediately after two washings with PBS, ROS formation was analyzed using a fluorometer (VICTOR 3, Perkin Elmer, Waltham, Mass, USA) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Western blotting - Cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 μg/μl aprotinin, 10 μg/μl leupeptin, 5 mM phenylmethanesulfonyluoride (PMSF), and 1 mM dithiothreitol (DTT) containing 1% Triton X-100). The supernatant extracts were centrifuged for 10 min at 12,000 × g at 4℃ and were subsequently employed for Western blot analysis. Equal amounts of protein were resolved on gradient (10%) SDS-PAGE gels (Invitrogen, Carlsbad, CA, USA) and electrophoretically transferred to nitrocellulose membranes. The membranes were subsequently blocked with 5% skim milk in TBST (20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.05% Tween 20) and incubated with the indicated antibodies. Western blotting was conducted using anti-human MMP-1 antibody (Calbiochem, San Diego, CA, USA). Western blotting proteins were visualized via enhanced chemiluminescence.

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR) - In order to assay for MMP-1 mRNA, total RNA was isolated as previously described [15]. RNA concentration was quantified via UV spectrophotometry at 260 nm and the purity was determined using the A_{260}/A_{280} ratio. All samples were reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Bioneer, Daejeon, Korea) and 30 pM oligo dT19 in a total reaction volume of 20 μl containing 5 × RT buffer (250 mM Tris-HCl, pH 8.3; 375 mM KCl; 15 mM $MgCl_2$, and 50 mM DTT), and 1 mM dNTPs. RT-PCR assays were conducted to specifically quantify the mRNA levels. In all assays, cDNA was amplified via a standardized program (5 min denaturing steps, 30 cycles of 30 seconds at 94℃, 30 seconds at 55℃, and 30 seconds at 72℃ melting point analysis in 1℃ steps and a final cooling step) using a Gene Amp PCR 2400 (Applied Biosystems, Foster City, CA, USA). The primers employed for β-act in were forward 5'-GGA CCT GAC AGA CTA CCT CA -3', reverse 5'-GTT GCC AAT AGT GAT GAC CT-3', and for MMP-1 they were forward 5'-GGT GAT GAA GCA GCC CAG-3' and reverse 5'-CAG TAG AAT GGG AGA GTC-3'.

Plasmid constructs - Genomic DNA was employed as a PCR template and primers at -2,270bp and + 30bp were

used to generate a fragment harboring a *Sac*I site at the 5' end and a *Hind*III site at the 3' end. PCR for the human MMP-1 promoter was carried out using a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA). The -2,270 MMP-1 promoter was subcloned into a pGEM T easy vector (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. White colonies that developed were checked for DNA ligation via mini preparation. Genotech (Daejeon, Korea) was contracted to sequence the ligated DNA. The -2,270 MMP-1 promoter subcloned into pGEM T easy vector was digested with *Sac*I and *Hind*III restriction enzymes. After SDS-PAGE, proteins were excised from the gel. The prepared pGL3-basic vector and -2,270 MMP-1 promoter were ligated in the same fashion as with the pGEM T easy vector system.

Luciferase assay for MMP-1 activity - Cells were seeded in 6-well plates at 3 x 10^5 cells/well with 2 ml of medium, then grown for 24 h. Transfection experiments were conducted with FuGENE-6 (Roche Molecular Biochemicals, Indianapolis, IN, USA) in accordance with the manufacturer's instructions. Transfection efficiency was measured via the X-gal staining method to optimize the conditions [16]. The plasmids used herein were 1 μg of test plasmid and 0.5 μg of pCMV β-galactosidase as an internal standard to adjust the transfection efficiency. Four hours after tansfection, the cells were washed twice with PBS and treated with 0.1, 1, or 10 μg/ml of *A. fistulosa* uronans in serum-free media overnight. The cells were then washed twice with PBS and irradiated with UVB at a dose of 15 mJ/cm^2. Luciferase activity was determined with a luminometer (TD 20/20, Promega, Sunnyvale, CA, USA) and luciferase activity was normalized for variation in transfection efficiency by dividing relative light units (RLU) by β-galactosidaseactivity.

Statistical analysis - Data were expressed as means ± SEM and were analyzed via analysis of variance (ANOVA) followed by Duncan's test. The significance level was set to $p<0.05$.

Results

A. fistulosa obtained from the Kurile Islands contained practically no fucoidans, or laminarans the polysaccharides regarded as characteristic of brown algae. The yield of uronans was 21.5% (from defatted seaweed weight). According to the NMR spectroscopy data, the water-soluble uronans isolated from *A. fistulosa* were enriched with mannuronic acid and the guluronic acid content was approximately 20-25%. The ^{13}C NMR spectrum of polymer contained signals for one anomeric carbon at 101.2 ppm (C1), one carbonyl carbon at 176.5 ppm (C6) and four ring carbons at 71.2 ppm (C2), 72.6 ppm (C3), 79.1 ppm (C4) and 77.1 ppm (C5) on the mannuronic acid. The low-field signal at 79.1 ppm showed the substitution of the sugar residue at position 4. The molecular weight of the polysaccharides was determined as described in the Materials and Methods section. These macromolecules had apparent molecular masses of 30-40 kDa.

As shown in figure (1), pretreatment with *A. fistulosa* uronans significantly attenuated an increase in intracellular ROS content, which was triggered by UVB irradiation. Uronans significantly inhibited UVB-induced ROS generation by 14.7% at 10 μg/ml compared to UVB irradiation alone ($p<0.05$).

The MMP-1 protein levels in the culture media were assessed via Western blotting. As shown in figure (2), *A. fistulosa* uronans significantly inhibited UVB-induced MMP-1 expression by 48.6% at 10 μg/ml compared to UVB irradiation alone ($p<0.05$) (figure 2). In order to determine the inhibitory effects of *A. fistulosa* uronans on UVB-induced MMP-1 mRNA expression at the transcription level, RT-PCR analysis was carried out using the total RNA isolated from the cells. As shown in figure (3), pretreatment with 1 and 10 μg/m luronans inhibited MMP-1 mRNA expression by 39.3% and 89.9% respectively, compared to UVB irradiation alone ($p<0.05$). The effect of *A. fistulosa* uronans on MMP-1 promoter activity was also evaluated. As shown in figure (4), uronans significantly inhibited UVB-induced MMP-1 promoter activity by 44.6% and 75.0% at respective concentrations of 1 and 10 μg/ml relative to UVB irradiation alone ($p<0.05$).

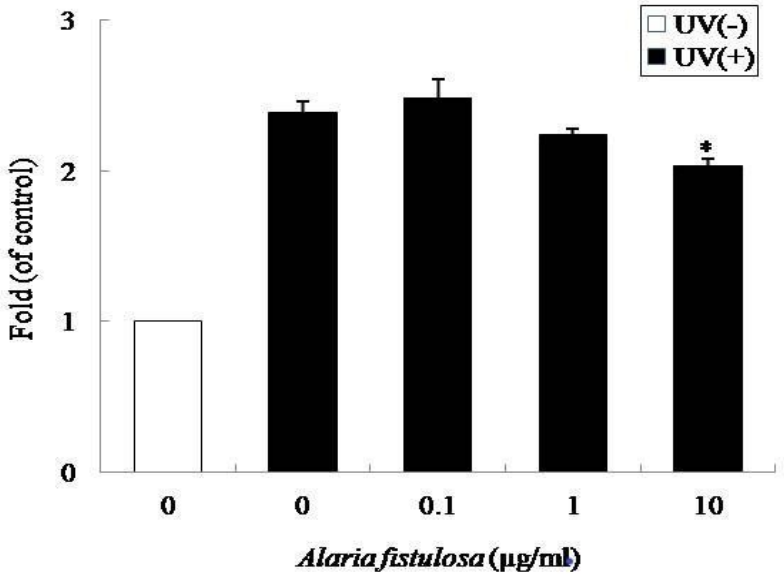

Figure 1: Inhibition of UVB-induced ROS generation by A. fistulosa uronans

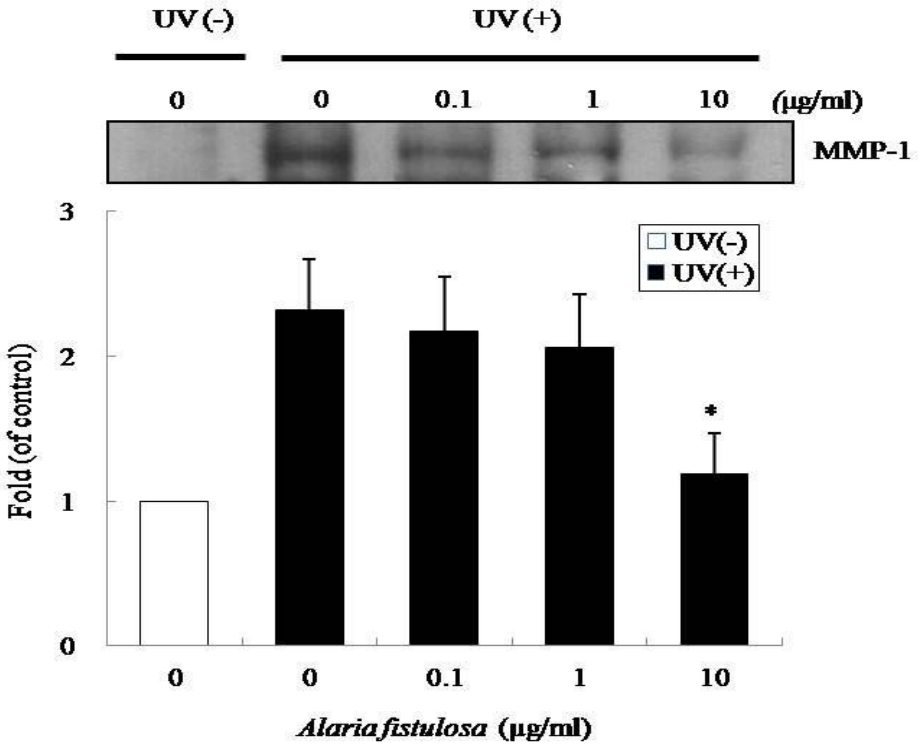

Figure 2: Inhibition of UVB-induced MMP-1 protein expression by A. fistulosa uronans

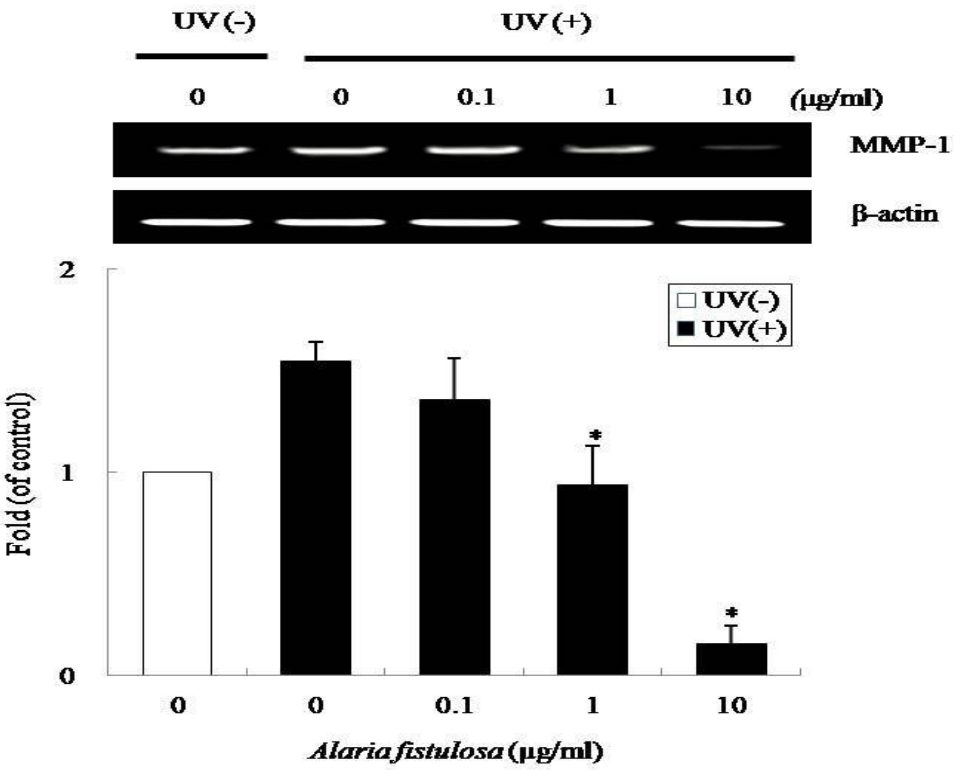

Figure 3: Effect of UVB-induced MMP-1 mRNA expression by A. fistulosa uronans

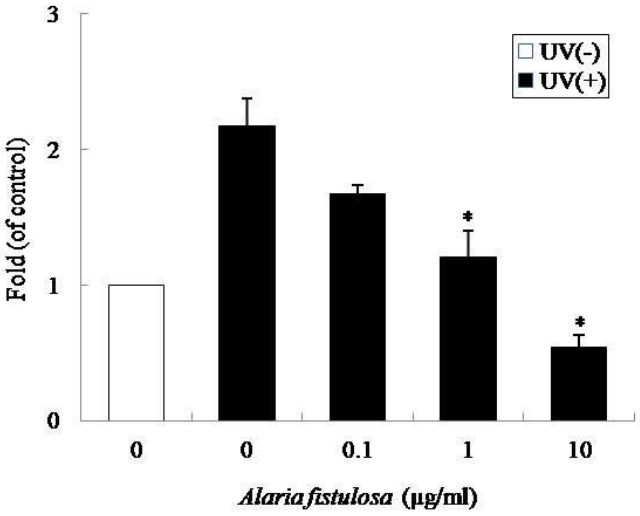

Figure 4: Inhibition of UVB-induced MMP-1 promoter activity by A. fistulosa uronans

Discussion

The brown algae occur abundantly throughout the world and are consumed as foods or food additives, while some of them have been developed into functional foods and new drugs, due to their safety and efficacy [17]. As different biological activities may depend on different structural fragments of the polysaccharide, the composition and structure of the fucoidan was investigated more thoroughly [18]. Little data is currently available regarding the content in seaweeds, as well as structure and properties of water-soluble natural polyuronans. Via a variety of *in*

vitro experiments, the effects of water-soluble uronans isolated from *A. fistulosa* on UVB-induced ROS generation and MMP-1 in HaCaT were evaluated.

UV radiation induces ROS generation in cultured human keratinocytes and fibroblasts *in vitro* and in human skin *in vivo*[19]. UVB-induced ROS is regarded as an important mediator of a variety of cutaneous pathologies, including cancer and skin aging [20]. Thus, strategies to prevent early pathological events induced by UV light may prove invaluable in efforts to achieve efficient and adequate photoprotection [21]. The authors found that *A. fistulosa* uronans markedly suppressed intracellular ROS generation after UVB irradiation. This result indicates that uronans may be capable of protecting the keratinocytes against UVB-induced oxidative damage. Some recent studies have reported that fucoidan can inhibit the synthesis and release of ROS, and also promote its clearance; this demonstrates fucoidan's anti-oxidative activity [22]. UV and ROS can activate the protein kinase cascade to stimulate the expression of the MMP gene. ROS also directly stimulates MMP zymogen activation by inducing the cleavage of the cysteine residue associated with the zinc active site. ROS is also suspected of being capable of enhancing MMP activities. Many previous studies focusing on the UVB-triggered induction of MMP-1 have identified components of the UVB-modulated signal transduction pathway(s), as well as the UVB irradiation-associated ROS generation [23].

UVB-triggered ROS generation induces MMP in photoaged skin, which affects connective tissue breakdown. MMP-1 is crucial to physiological or pathological processes since it has the unique property of initiating the cleavage of native fibers of collagens type I and III, which represent more than 90% of the ECM of skin and the subsequent formation of wrinkles [8]. Recent studies have demonstrated that hairless mice exposed to UVB develop wrinkled skin and significantly enhanced MMP-1 mRNA expression. However, those studies have also shown that the inhibition of MMP-1 activities via a specific MMP inhibitor suppress UVB-induced wrinkle formation. This evidence suggests that MMP-1 performs a major role in the process of photoaging. Therefore, agents that inhibit MMP-1 activity may prove beneficial for the maintenance of healthy skin by preventing the degradation of the dermal matrix.

Recently, fucoidan was evaluated for its potential biological activities in anti-skin aging. Active compounds from 29 marine natural products were screened for the ability to inhibit MMP-1 expression in human dermal fibroblasts, and were successful in identifying eckol and dieckol from *Ecklonia stolonifera* as potent inhibitors of MMP-1 expression [24]. Using *in vitro* models of dermal wound repair, fucoidan modulates the effect of TGF-1 on fibroblast proliferation and wound repopulation during wound repair [25]. Polysaccharides from brown algae stimulate dermal fibroblast proliferation and extracellular matrix deposition *in vitro*, to control important parameters involved in connective tissue breakdown in human skin [26]. Additionally, other recent studies, specifically involving the developing embryos of sea urchins, have reported a stimulatory effect of polyuronans; the results of the same study suggested that polyuronans from *A. fistulosa* might prove an interesting candidate as an immunostimulant agent [12]. This study, as well as other similar studies, underline the possibility that agents for the prevention and treatment of skin aging might be developed from such natural sources. However, no specific *in vitro* investigations of the effects of polyuronans on MMP-1 expression have been conducted thus far. Herein, we evaluated the effects of *A. fistulosa* uronan on MMP-1 expression. UVB-irradiation induced MMP-1 expression in HaCaT, and, as expected, uronans significantly inhibited UVB-induced MMP-1 mRNA and protein expression in a dose-dependent manner compared to UVB irradiation alone.

MMP-1 mRNA and protein levels are associated with MMP-1 gene expression [27]. It has been previously suggested that MMP-1 gene expression may be regulated in a cell-type- specific manner, that includes transcriptional and post-transcriptional mechanisms [28]. Other studies have also reported that MMP-1 activity is stringently regulated at three levels: promoter, activation of proenzyme, and inhibition of active enzyme [16]. The regulation of MMPs occurs primarily at transcriptional level [29]. We noted that UVB-induced MMP-1 promoter activity was inhibited more profoundly by *A. fistulosa* uronans than by UVB irradiation alone.

The obtained data in this study demonstrate that *A. fistulosa* uronans may prevent UVB-induced MMP-1 expression transcriptionally, translationally, and by affecting protein function. It could be suggested that *A. fistulosa* uronans may function as therapeutic agents that can remediate, and perhaps even curtail, photoaging of the skin. Further, *in*

vitro studies to elucidate the pathways via which this inhibition occurs are clearly warranted.

Conclusion

Pretreatment with water-soluble uronans (the low-molecular-weight analogues of alginic acids) isolated from *A. fistulosa* significantly inhibited UVB-induced ROS generation and MMP-1 expression. Uronans significantly reduced MMP-1 mRNA expression and inhibited UVB-induced MMP-1 promoter activity. *A. fistulosa* uronans may be a potential therapeutic agent for the prevention and treatment of skin photoaging.

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