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Effect of *Costaria costata* Fucoidan on Expression of Matrix Metalloproteinase-1 Promoter, mRNA, and Protein

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Fucoidans are sulfated fucosylated polymers from brown algae cell walls. We assessed the inhibitory effects of *Costaria costata* fucoidan on UVB-induced MMP-1 promoter, mRNA, and protein expression *in vitro* using the immortalized human keratinocyte (HaCaT) cell line. Pretreatment with fucoidan significantly inhibited MMP-1 protein expression compared to UVB irradiation alone. Fucoidan significantly reduced MMP-1 mRNA expression and inhibited UVB-induced MMP-1 promoter activity by 37.3%, 53.3%, and 58.5% at 0.01, 0.1, and 1 μg/mL, respectively, compared to UVB irradiation alone. *C. costata* fucoidan may be a potential therapeutic agent to prevent and treat skin photoaging.

Brown seaweeds produce different polysaccharides including alginates, laminarans, and fucoidans.[1,2] The latter usually contain large proportions of L-fucose and sulfate, together with minor amounts of other sugars such as xylose, galactose, mannose, and glucuronic acid.[2,3] Biological activities attributed to the polysaccharides include anticoagulant,[4] antithrombotic,[5] antitumoral,[6−8] and antiviral[9] activities. Fucoidan also inhibits tube formation of human endothelial cells[10] as well as tumor progression through the modulation of host immune systems.[11−13] An *in vivo* study of the antidiabetic potential of the brown algae *Petalonia binghamiae* reported that dietary administration to streptozotocin-induced diabetic mice significantly lowered blood glucose levels and improved glucose tolerance.[14] We also previously reported that fucoidan inhibits ultraviolet B (UVB)-induced matrix metalloproteinase-1 (MMP-1) expression in human skin fibroblasts at the mRNA and protein levels.[15] Our study indicated that fucoidan may be a potential therapeutic agent to prevent and treat skin photoaging.[15]

Brown seaweeds have been extensively studied due to their numerous interesting biological activities of the diverse compounds that, to date, have not been isolated from any other source. Of particular interest to a global population that is becoming more elderly is the potential of natural compounds derived from brown seaweeds to lessen the effects of aging of skin.

The skin dermis contains predominantly type I and type III collagen, elastin, proteoglycans, and fibronectin. Because collagen fibrils and elastin are responsible for the strength and resiliency of skin, their disarrangement during photoaging causes the skin to appear aged. Excessive matrix degradation by UV-induced MMP-1 secreted by various cells, including keratinocytes, fibroblasts, and inflammatory cells, contributes substantially to the connective tissue damage that occurs during photoaging,[16−19] through cleavage of fibrillar type I and III collagen in skin at a single site within the collagen's central triple helix.[20] This suggests that the development of MMP-1 inhibitors may represent a promising antiaging strategy for skin. Indeed, flavonoid compounds such as naringenin, apigenin, wogonin, kaempferol, and quercetin can regulate MMP-1 expression.[21]

Costaria costata is a brown algae in the order Laminariales, family Laminariaceae. It has been found in the Bering Sea, in the northwest Pacific off the northern end of Honshu Island, Hokkaido, and the Sea of Japan bounding Russia and Korea. Herein, we assessed the inhibitory effects of *C. costata* fucoidan on UVB-induced MMP-1 expression *in vitro*.

Results and Discussion

C. costata samples were collected during May 2006 from Troiza Bay, Sea of Japan. The sulfated fucoidans were purified as previously described.[22,23] The chemical compositions of the novel fucoidans used in this study are provided in Table 1. The fucoidans were structurally heterogeneous due to variations in their carbohydrate contents and noncarbohydrate subsistent (sulfate and acetyl groups).

To examine the effect of UVB irradiation, cells were exposed to 15 mJ/cm^2 UVB, and their viabilities were measured 24 h later using a standard [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT] assay. UVB exposure decreased cell viability (Table 2). To further clarify the effects of *C. costata* fucoidan on the viability of UVB-irradiated cells, fucoidan was added at concentrations of 0.01, 0.1, and 1 μg/mL. Fucoidan concentrations of 0.01 and 0.1 μg/mL increased cell viability by 1.77% and 4.94%, respectively, compared to UVB irradiation alone, while viability was slightly decreased at 1 μg/mL. Fucoidan was mildly toxic at the highest concentration.

After treatment for 24 h with 0.01, 0.1, or 1 μg/mL fucoidan, the cells were mock-treated or UVB irradiated (15 mJ/cm^2), washed with phosphate-buffered saline (PBS), and incubated for a further 72 h. Fucoidan treatment significantly inhibited the expression of MMP-1 in a dose-dependent manner (Figure 1). Fucoidan inhibited UVB-induced MMP-1 expression by 41.8% at 0.01 μg /mL, 57.7% at 0.1 μg /mL, and 70% at 1 μg /mL compared to UVB irradiation alone ($p < 0.05$). The effect of *C. costata* fucoidan on skin aging has not been reported to date. Senni et al.[24] reported that polysaccharides from brown algae are able to stimulate dermal fibroblast proliferation and extracellular matrix deposition *in vitro*, important parameters involved in connective tissue breakdown. Joe et al.[25] screened active compounds from 29 marine natural products for the ability to inhibit MMP-1 expression in human dermal fibroblasts and were successful in identifying eckol and dieckol from *Ecklonia stolonifera* as being potent inhibitors of MMP-1 expression. Such studies highlight the possibility of developing agents for the prevention and treatment of skin aging.

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Table 1. Yields and Composition of *C. costata* Fucoidan

seaweed source	yield, % of dry biomass	$NaSO_3$ (%)	uronic acids (%)	neutral monosaccharides (%)				
				fucose	mannose	galactose	xylose	glucose
C. costata	0.3	5.28	28.2	23.4	8.2	5.1	2.1	4.0

Table 2. Effects of *C. costata* Fucoidan on Cell Viabilities under UVB Irradiation[a]

		fucoidan (μg/mL)			
	dose	0	0.01	0.1	1
UVB (mJ/cm^2)	0	$100.00^b \pm 10.98$	$110.45^a \pm 15.92$	$117.78^a \pm 12.33$	$96.63^b \pm 11.81$
	15	$85.58^a \pm 10.99$	$87.35^a \pm 2.91$	$90.52^a \pm 5.18$	$76.52^b \pm 8.51$

[a] The cells were pretreated with fucoidan (0, 0.01, 0.1, 1 μg/mL) prior to UVB irradiation and incubated for 24 h. Cell viability was measured by MTT assay, as described in the Experimental Section, and was calculated in terms of relative values. Each value represents mean ± SEM ($n = 15$). All values are normalized relative to the zero dose/zero UVB exposure samples. Values in a row with different letters a and b are significantly different ($p < 0.05$).

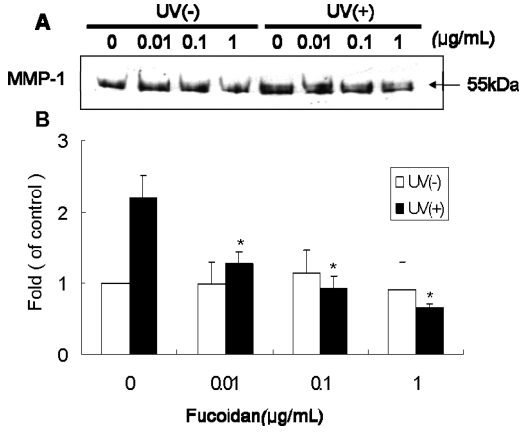

Figure 1. Inhibition of UVB-induced MMP-1 protein expression by *C. costata* fucoidan in HaCaT immortalized human keratinocyte cells. (A) After treatment with fucoidan for 24 h, cells were mock-treated or irradiated with UVB (15 mJ/cm^2). The cells were washed with PBS and further incubated for 72 h. MMP-1 expression was determined in culture media by western blotting. (B) Data of MMP-1 expression quantified by densitometry. Values are presented as mean ± SEM of three independent experiments. *$p < 0.05$ compared to UVB irradiation alone group.

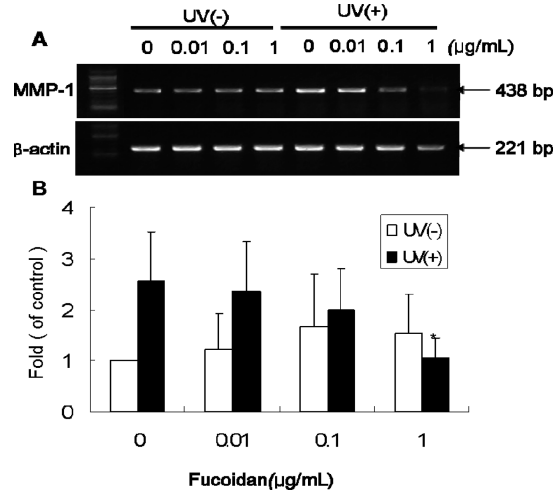

Figure 2. Effect of UVB-induced MMP-1 mRNA expression by *C. costata* fucoidan in HaCaT immortalized human keratinocyte cells. (A) PBS was added to quiescent cells prior to UVB exposure (15 mJ/cm^2). After UVB irradiation, the cells were washed with PBS and further incubated for 24 h. MMP-1 mRNA was determined by RT-PCR. (B) Data of MMP-1 expression quantified by densitometry. Values are presented as mean ± SEM of three independent experiments. *$p < 0.05$ compared to UVB irradiation alone group.

To study the inhibitory effect of fucoidan on UVB-induced MMP-1 mRNA expression at the transcription level, reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed using total RNA isolated from the cells. As shown in Figure 2, pretreatment with 0.01, 0.1, or 1 μg/mL fucoidan inhibited MMP-1 expression by 7.4%, 22%, and 59.1%, respectively, compared to UVB irradiation alone ($p < 0.05$). When the cells were treated with fucoidan without UVB irradiation, the expression of MMP-1 mRNA was not changed compared to a control.

The hMMP-1 promoter/luciferase reporter plasmids (−2270-hMMP-1/Luc, −1225hMMP-1/Luc, and −546hMMP-1/Luc) used in this study contain fragments of promoter DNA linked to the luciferase reporter (Figure 3). We tested the UVB response ability of each promoter construct (Figure 4). Plasmids −1225hMMP-1/Luc and −546hMMP-1/Luc produced only a minimal transcriptional response, while −2270hMMP-1/Luc showed increased transcriptional activity. Henceforth, studies of the inhibitory effect on MMP-1 promoter used only −2270hMMP-1/Luc. The effect of *C. costata* fucoidan on MMP-1 promoter activity was also assessed. Fucoidan significantly inhibited UVB-induced MMP-1 promoter activity by 37.3%, 53.3%, and 58.5% at respective concentrations of 0.01, 0.1, and 1 μg /mL compared to UVB irradiation alone (Figure 5). Recently it was suggested that MMP-1 gene expression may be regulated in a cell-type specific manner that includes transcriptional and post-transcriptional mechanisms.[26] Consistent with this, the activity of MMP-1 is stringently regulated at three levels, promoter, activation of proenzyme, and inhibition of the active enzyme,[27] but MMPs may be regulated primarily at the level of transcription activity.[28] We confirmed that MMP-1 activation is regulated by UVB and observed that UVB-induced MMP-1 promoter activity was inhibited by *C. costata* fucoidan compared to UVB irradiation alone. In an earlier study, the binding sites for activator protein-1 (AP-1) were found to be important to MMP-1 promoter regulation,[29] and further studies have indicated that suppressors of AP-1 inhibit MMP-1 promoter regulation.[30−33] Additional studies are needed to identify the critical regulatory-transcriptional factors and the MMP-1 promoter regulatory regions controlled by fucoidan.

Our data indicate that *C. costata* fucoidan may prevent UVB-induced MMP-1 expression at promoter, mRNA, and protein levels. We suggest that *C. costata* fucoidan is a potential therapeutic agent for the prevention and treatment of photoaging of the skin. Further, *in vitro* studies will be necessary to elucidate the pathways of inhibition.

Experimental Section

Acute Exposure of Transfected and Wild-Type Human Keratinocyte to UVB Radiation. The immortalized human keratinocyte cell line, HaCaT,[34] was obtained from Cell Line Service (Eppelheim, Baden-Württemberg, Germany). The cells were plated in 100 mm tissue culture

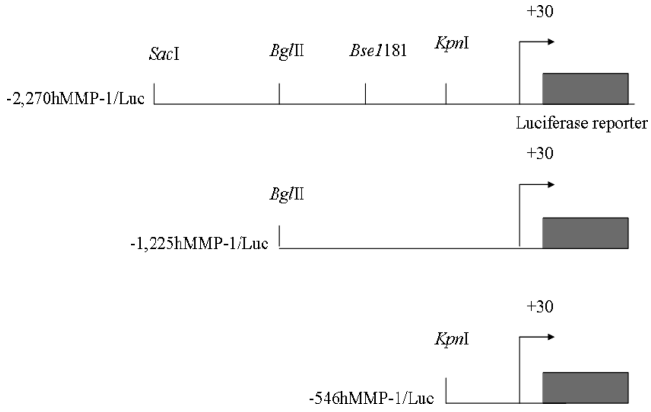

Figure 3. Structure of the human MMP-1 promoter/luciferase reporter plasmids (−2270hMMP-1/Luc, −1225hMMP-1/Luc, and −546hMMP-1/Luc). The plasmids contain fragments of promoter DNA linked to the luciferase reporter. Plasmid −2270hMMP-1/Luc has variously located restriction sites, which were used to generate the clones as −1225hMMP-1/Luc and −546hMMP-1/Luc.

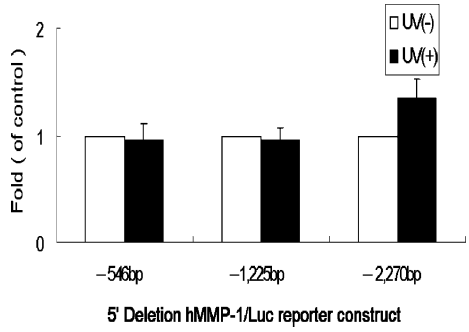

Figure 4. Transcriptional response based on MMP-1 promoter region by UVB in HaCaT immortalized human keratinocyte cells. UVB response of each promoter construct was ascertained. After transfection, cells were washed twice with PBS and incubated overnight. PBS was added to quiescent cells prior to UVB exposure (15 mJ/cm^2). After UVB irradiation, cells were washed with PBS and further incubated for 24 h. MMP-1 promoter activity was determined by luminometry. Values are presented as mean ± SEM of three independent experiments.

dishes and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin−streptomycin (all from GIBCO-BRL, Grand Island, NY). *C. costata* fucoidan was dissolved in distilled water. For treatment, the cells were maintained in culture media with 0.5% FBS, followed by treatment with fucoidan from *C. costata* for 24 h. The cells were rinsed twice with PBS, and all UVB irradiation exposures were performed following the addition of 2 mL of PBS. Immediately after irradiation, the cells were incubated in serum-free fresh culture media containing *C. costata* fucoidan. The UV light source originated from 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.

Polysaccharide Extraction. *C. costata* samples were collected in the Troiza Bay, Sea of Japan, at the Sea Experimental Station of Pacific Institute of Bioorganic Chemistry, Far-East Branch, Russian Academy of Sciences (Primorski Krai, Hasanski region). The isolation and separation of water-soluble polysaccharides were carried out as previously described.[22,23] Fresh seaweed (1 kg) was initially washed with H_2O, then twice treated with EtOH (1.3 kg) at 40 °C for 3 h. Samples of the defatted, dried, and powdered (1 mm) algal fronds (50 g) were extracted twice with 0.1 M HCl for 3 h at 20 °C. The polysaccharide fraction was concentrated, dialyzed, and lyophilized.

Analytical Procedures. Neutral carbohydrates were quantified by the phenol-sulfuric acid method.[35] Monosaccharide composition was

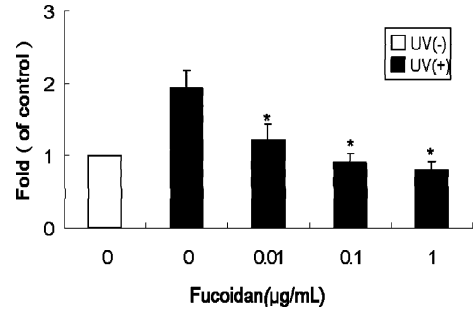

Figure 5. Inhibition of UVB-induced MMP-1 promoter activity by *C. costata* fucoidan in HaCaT immortalized human keratinocyte cells. Fragments ranging from 546 to 1225 bp gave only a minimal transcriptional response, while the −2270hMMP-1/Luc showed increased transcriptional activity. Henceforth, studies of the inhibitory effect on MMP-1 promoter used only the −2270hMMP-1/Luc. The plasmids used were 1 μg of the 2270 bp clone of the MMP-1 promoter and 0.5 μg of pCMV-β galactosidase as internal standards for the adjustment of transfection efficiency. After transfection, the cells were washed twice with PBS and treated with fucoidan overnight. PBS was added to quiescent cells prior to UVB exposure (15 mJ/cm^2). After UVB irradiation, cells were washed with PBS and further incubated for 24 h. MMP-1 promoter activity was determined by luminometry. Values are presented as mean ± SEM of four independent experiments. *$p < 0.05$ compared to UVB irradiation alone group.

determined by HPLC with an LC-5001 carbohydrate analyzer equipped with a Durrum DA-X8-11 column (385 × 3.2 mm) (Biotronik, Berlin, Germany), bicinhoniate assay, and a C-R2 AX integrating system (Shimadzu, Kyoto, Japan) after hydrolysis by 2 M TFA at 100 °C for 6 h. Sulfate groups in the polysaccharides were quantified by the titrometric method.[36] Uronic acids were quantified using a CECIL-2021 spectrophotometer (Cecil Instruments, Cambridge, England) with 3,5-dimethylphenol and sulfuric acid.[37]

Western Blotting. Cells were lysed with a 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 phenylmethanesulfonyl fluoride (PMSF), and 1 mM DTT containing 1% Triton X-100). The supernatant extracts were centrifuged at 12000*g* for 10 min at 4 °C to remove debris, and the resulting supernatant was used for western blot analysis. Equal amounts of protein were resolved using gradient 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) (Invitrogen, Carlsbad, CA) 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 performed using anti-human MMP-1 mouse antibody as primary antibody (Calbiochem, San Diego, CA, 1:100 dilution in TBST + 3% skim milk) and goat anti-mouse IgG-horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Santa Cruz, CA, 1:2000 dilution in TBST + 3% skim milk) as secondary antibody. The proteins were visualized by enhanced chemiluminescence.

RNA Extraction and RT-PCR. To assay for the MMP-1 mRNA, total RNA was isolated as described previously.[38] RNA concentration was quantified by UV spectrophotometry at 260 nm, and the purity was determined using the A_{260}/A_{280} ratio. All samples were reverse-transcribed using moloneymurine 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 was performed to specifically quantify the mRNA level. In all assays, the cDNA was amplified using a standardized program (5 min denaturing steps, 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C, melting point analysis in 1 °C steps, and a final cooling step) using a Gene Amp PCR 2400 (Applied Biosystems, Foster City, CA). The primers used for β-actin 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 used as a PCR template along with primers at −2270 bp and +30 bp to generate a fragment containing a 5′ *Sac*I site and a 3′ *Hind*III site. PCR for human MMP-1 promoter was performed in a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA). PCR of the MMP-1 promoter consisted of 95 °C for 1 min followed by 30 cycles of 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min. The PCR products were purified by a QIAquick PCR purifiction kit (Qiagen, Hilden, Germany), and enzyme digestion by *Sac*I and *Hind*III was performed. After gel electrophoresis, each 2.3 kb DNA fragment was extracted from gel by use of a gel extraction kit (Qiagen). The −2300 MMP-1 promoter was subcloned into pGEM T Easy Vector (Promega, Madison, WI) according to the manufacturer's instructions. Select white-appearing colonies were checked to confirm vector ligation by mini gel preparation and sequencing of the ligated DNA (sequencing was contracted to Genotech, Daejeon, Korea). The pGL3-basic vector was digested by *Sac*I at 37 °C overnight. The DNA was precipitated in exchange reaction buffer, and gel electrophoresis was performed. DNA of 4.8 kb size was extracted as described above. The DNA was digested by *Hind*III at 37 °C overnight and precipitated. A −2300 MMP-1 promoter subcloned into pGEM T Easy Vector (Promega) was digested using *Sac*I and *Hind*III. The resulting fragments were separated by gel electrophoresis and were extracted with a gel extraction kit. The prepared pGL3-basic vector and −2300 MMP-1 promoter were similarly ligated. In addition to −1225hMMP-1/Luc and −546 hMMP-1/Luc, −2270hMMP-1/Luc was cut with *Bgl*II and *Kpn*I at the 5′ end. After restriction enzyme digestion, each deletion construct was ligated.

Luciferase Assay for MMP-1 Activity. Cells were seeded in wells of six-well plates at 3×10^5 cells/well with 2 mL of medium and grown for 24 h. Transfection experiments were carried out with the FuGENE-6 (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. Transfection efficiency was measured by the X-gal staining method[39] to optimize the conditions. The plasmids used were 1 μg of test plasmid and 0.5 μg of pCMV-β-galactosidase as an internal standard to adjust transfection efficiency. Four hours after the transfection, the cells were washed twice with PBS and treated with 0.01, 0.1, or 1 μg/mL *C. costata* fucoidan in serum-free medium 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 TD 20/20 luminometer (Promega, Sunnyvale, CA), and luciferase activity was normalized for variation in transfection efficiency by dividing relative light units (RLU) by β-galactosidase activity.

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

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