

Effect of *Fucus evanescens* Fucoidan on Expression of Matrix Metalloproteinase-1 Promoter, mRNA, Protein and Signal Pathway

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Fucoidans are sulfated fucosylated polymers from the cell wall of brown algae. We assessed the effects of *Fucus evanescens* fucoidan on ultraviolet-B (UVB)-induced expression of matrix metalloproteinase-1 (MMP-1) protein, mRNA, and promoter, and the phosphorylation of mitogen-activated protein kinases *in vitro* using an immortalized human keratinocyte cell line. Pretreatment with 10 and 100 µg/ml fucoidan significantly inhibited UVB-induced MMP-1 protein, mRNA and promoter activity, compared to UVB irradiation alone. Extracellular signal regulated kinase activation was markedly inhibited by treatment with fucoidan, though c-JUN N-terminal kinase activity and p38 activation were only marginally affected by fucoidan. *F. evanescens* fucoidan may be a potential therapeutic agent for the prevention and treatment of skin photoaging.

Key words : Matrix metalloproteinase-1, fucoidan, ultraviolet B, photoaging, skin

Introduction

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. Recently, it was suggested that excessive matrix degradation by ultraviolet (UV) induced matrix metalloproteinase-1 (MMP-1) secreted by various cells including keratinocytes, fibroblasts, and inflammatory cells contributes substantially to the connective tissue damage that occurs during photoaging [7,11,27], through cleavage of fibrillar collagen (type I and III in skin) at a single site within its central triple helix [13]. This evidence suggests that the expression of MMP-1 and the down-regulation of type I collagen synthesis play major roles in the process of photoaging. In the absence of perfect repair, MMP-1 mediated collagen damage is expected to accumulate with each successive UV exposure. Such cumulative collagen damage is likely a major contributor to the phenotype of photoaged human skin [30]. This suggests that

the development of MMP-1 inhibitors may represent a promising anti-aging strategy for skin. Indeed, flavonoid compounds such as naringenin, apigenin, wogonin, kaempferol, and quercetin can regulate MMP-1 expression [24].

Since fucoidan was first isolated nearly a century ago [20], the structures of fucoidans from different brown seaweeds have been investigated. Most fucoidans have a complex composition [23]. For example, fucoidans from the brown seaweed *Fucus evanescens* C. Ag consists of fucose, sulfate and acetate [4], and has a linear backbone of alternating 3- and 4-linked alpha-L-fucopyranose 2-sulfate residues, with additional sulfate occupying position 4 in some 3-linked fucose residues [2]. In spite of increasing study on the various biological activities of fucoidan, the effects of *F. evanescens* fucoidan on skin photoaging have not been investigated.

Herein, we assessed the inhibitory effects of *F. evanescens* fucoidan on UVB-induced MMP-1 expression *in vitro* and elucidated the pathways of inhibition.

Materials and Methods

Acute exposure of transfected and wild type human keratinocyte to UVB radiation

The immortalized human keratinocyte cell line, HaCaT

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[5], was obtained from Cell Line Service (Eppelheim, Baden-Württemberg, 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 from GIBCO-BRL, Grand Island, NY). *F. evanescens* fucoidan was dissolved in distilled water. For treatment, the cells were maintained in 0.5% FBS supplemented DMEM, followed by treatment with *F. evanescens* fucoidan for 24 hr. The cells were rinsed twice with phosphate buffered saline (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 *F. evanescens* fucoidan. The UV light source was a Philips TL 20 W/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/cm² dose of UVB light.

Polysaccharide extraction

F. evanescens samples were collected from Paramushir Island, Sea of Okhotsk. The isolation and separation of water-soluble polysaccharides were carried out by modified methods [18,33]. Fresh or deep-frozen seaweed (1 kg) was initially and successively treated with ethanol, acetone, and chloroform. Samples of defatted, dried, and powdered algal fronds (200 g) were extracted twice with 0.1 M HCl (2.5 l) for 2 hr at 60°C. The extracts were concentrated to 20% of their original volume by ultrafiltration with the use of a 3 kDa membrane (Millipore, Beverly, MA), dialyzed and polysaccharides were precipitated with four volumes of 96% ethanol. The precipitates were washed with 96% ethanol and acetone and air-dried.

Anion exchange chromatography

A solution of polysaccharide in 0.1 M NaCl (2.3 g in 50 ml) was applied onto a DEAE-cellulose column (Cl⁻ form, 3×21 cm; Sigma-Aldrich, St. Louis, MO) equilibrated with 0.1 M NaCl. The column was then successively eluted with 0.1, 0.5, 1, and 2 M NaCl, each time until the disappearance in the eluent of positive reaction for carbohydrates by the phenol-sulfuric acid method [10]. The corresponding polysaccharide fractions were concentrated by ultrafiltration (1 kDa cutoff), dialyzed, and lyophilized.

Analytical procedures

Total carbohydrates were quantified by the phenol-sulfu-

ric acid method [10]. Monosaccharides composition was determined by high-pressure liquid chromatography with a LC-5001 carbohydrate analyzer (Durrum DA-X8-11 column, 385 x 3.2 mm; Biotronik, Berlin, Germany), bichoninate assay, and a C-R2 AX integrating system (Shimadzu, Kyoto, Japan) after hydrolysis by 2 M trifluoroacetic acid (6 hr, 100 °C). The content of protein was determined by the method of Lowry *et al.* [25]. Sulfate group determination was carried out using the BaCl₂ gelatin method [9].

¹³C-Nuclear Magnetic Resonance (NMR)

Spectra of ¹³C-NMR for solutions of substances in deuterium oxide were obtained on an Avance DPX-500 NMR spectrometer (Bruker, Berlin, Germany) with a working frequency of 75.5 MHz at 60°C.

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, and 1 mM dithiothreitol (DTT) containing 1% Triton X-100). The supernatant extracts were centrifuged at 12,000× *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 the anti-human MMP-1 antibody (1:250 dilution) (Calbiochem, San Diego, CA), anti-phospho-c-Jun N terminal kinase (JNK; 1:250 dilution), anti-phospho-extracellular signal related kinase (ERK; 1:500 dilution), anti-phospho-p38 mitogen-activated protein kinase (p38 MAPK; 1:250 dilution), anti-total-JNK (1:500 dilution), anti-total-ERK (1:500 dilution), and anti-total-p38 (1:500 dilution) (all from Cell Signaling Technology, Danvers, MA). Western blotting 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 [6]. 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 moloney murine leukemia virus reverse transcriptase (Bioneer, Daejeon, Korea) and 30 pM oligo dT19 in a total reaction volume of 20 μ l containing 5 x 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, cDNA was amplified using a standardized program (5 min denaturing steps, 30 cycles of 30 sec at 94°C, 30 sec at 55°C, and 30 sec 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 -2,270 bp and +30 bp to generate a fragment containing a 5' *Sad* site and a 3' *HindIII* site. PCR for human MMP-1 promoter was performed in a GeneAmp[®] PCR System 2700 (Applied Biosystems). 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 purification kit (Qiagen, Valencia, CA) and enzyme digestion by *Sad* and *HindIII* was performed. After gel electrophoresis, each 2.3 kb DNA fragment was extracted from gel by use of a gel extraction kit (Qiagen). The -2,300 MMP-1 promoter was subcloned into pGEM[®] T easy vector (Promega, Madison, WI) according to the manufacturer's instructions. White-appearing colonies were selected and 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 *Sad* at 37°C overnight. The DNA was precipitated in exchange reaction buffer and gel electrophoresis was performed. DNA 4.8 kb in size was extracted as described above. The DNA was digested by *HindIII* at 37°C overnight and precipitated. A -2,300 MMP-1 promoter subcloned into pGEM[®] T easy vector (Promega) was digested using *Sad* and *HindIII*. The resulting fragments separated by gel electrophoresis and were extracted with a gel extraction kit. The prepared pGL3-basic vector and -2,300 MMP-1 promoter were sim-

ilarly ligated.

Luciferase assay for MMP-1 activity

Cells were seeded in wells of 6-well plates at 3×10^5 cells/well with 2 ml of medium and grown for 24 hr. 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 [32] to optimize the conditions. The plasmids used were 1 μ g of test plasmid and 0.5 μ g 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 1, 10, or 100 μ g/ml of *F. evanescens* fucoidan in serum-free medium overnight. The cells were then washed twice with PBS and irradiated with UVB at a dose of 15 mJ/cm². 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 were expressed as the mean \pm SEM and were analyzed by analysis of variance (ANOVA) followed by Duncan's test. The significance level was set to $p < 0.05$.

Results

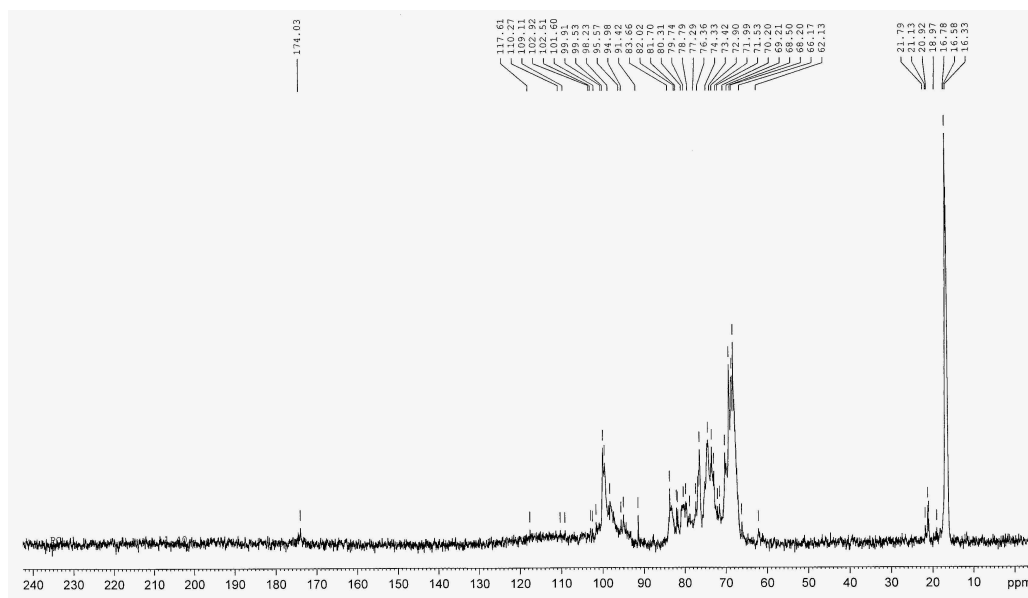
Purification of *F. evanescens* fucoidan and cell viability under action of *F. evanescens* fucoidan

Polysaccharides were isolated from the seaweed *F. evanescens* collected from its natural habitat. Seaweed was initially defatted with ethanol and acetone to remove pigments and other low-molecular weight compounds. Water soluble polysaccharides were then extracted from defatted biomass with diluted HCl at 60°C. The extract was concentrated and dialyzed, and polysaccharides were precipitated with ethanol. The resulting crude fucoidan was purified and fractionated by ion-exchange chromatography on DEAE-cellulose using aqueous NaCl of increasing concentration as eluent. The fucoidan eluted with 2 M NaCl was essentially a homofucan sulfate containing fucose and only traces of other monosaccharide constituents (Table 1). Chemical analysis of the fucoidan from *F. evanescens* revealed a 21% of sulfate ester and ¹³C NMR revealed a complex spectrum (Fig.

Table 1. Yields and composition of fucoidan from *F. evanescens* obtained by ion-exchange chromatography

Seaweed source	Yield*, %	SO ₃ Na, %	Neutral monosaccharides, mol %				
			Glucose	Fucose	Galactose	Mannose	Xylose
<i>F. evanescens</i>	25.3	21	0	1	0.01	0.07	0.03

*From crude polysaccharide.

Fig. 1. ¹³C NMR spectrum of fucoidan from *F. evanescens*

1). It contained several intense signals in the anomeric (97-102 ppm) and high-field (16.5-16.7 ppm) regions typical of alpha-fucopyranosides. The signals at 19-20 ppm confirmed the presence of *O*-acetyl groups.

Effect of *F. evanescens* fucoidan on UVB induced MMP-1 expression

After treatment for 24 hr with 1, 10, or 100 µg/ml fucoidan, cells were mock-treated or UVB irradiated (15 mJ/cm²), washed with PBS, and incubated for a further 72 hr. Fucoidan treatment significantly inhibited the expression of MMP-1 in a dose-dependent manner (Fig. 2). Fucoidan inhibited UVB-induced MMP-1 expression by 33.0% at 10 µg/ml and 57.4% at 100 µg/ml compared to UVB irradiation alone ($p < 0.05$).

Effect of *F. evanescens* fucoidan on UVB induced MMP-1 mRNA expression

To study the inhibitory effect of fucoidan on UVB-induced MMP-1 mRNA expression at the transcription level, RT-PCR analysis was performed using total RNA isolated from the cells. As shown in Fig. 3, pretreatment with 10 or 100 µg/ml fucoidan inhibited MMP-1 expression by 28.4% and 59.7%,

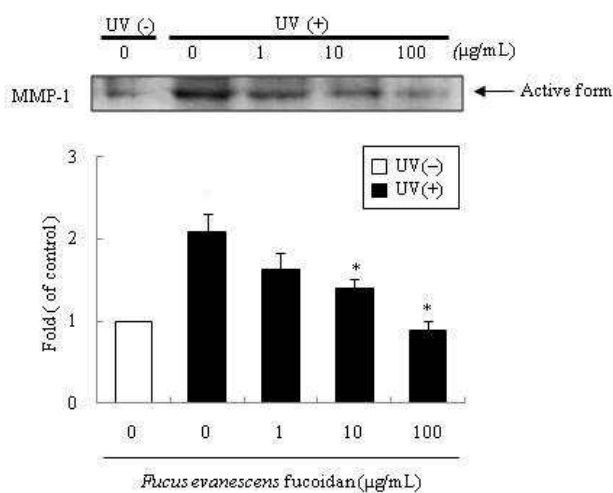


Fig. 2. Inhibition of UVB-induced MMP-1 protein expression by *F. evanescens* fucoidan in human keratinocyte cells. After treatment with fucoidan for 24 hr, HaCaT cells were mock-treated or irradiated with UVB (15 mJ/cm²). The cells were washed with PBS and further incubated for 72 hr. MMP-1 expression was determined in culture medium by Western blotting. Each bar of the lower figure shows data of MMP-1 expression quantified by densitometry. Values are presented as means±SEM of 5 independent experiments. * $p < 0.05$ compared to UVB irradiation alone group.

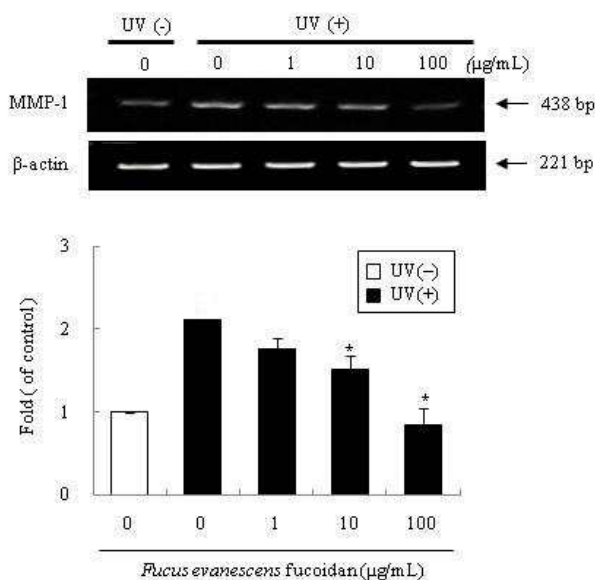


Fig. 3. Effect of UVB-induced MMP-1 mRNA expression by *F. evanescens* fucoidan in human keratinocyte cells. PBS was added to quiescent HaCaT cells prior to UVB exposure (15 mJ/cm^2). After UVB irradiation, cells were washed with PBS and further incubated for 24 h. MMP-1 mRNA was determined by RT-PCR. Each bar of the lower figure shows data of MMP-1 expression quantified by densitometry. Values are presented as means \pm SEM of 5 independent experiments. * $p < 0.05$ compared to UVB irradiation alone group.

respectively, compared to UVB irradiation alone ($p < 0.05$).

Effect of *F. evanescens* fucoidan on UVB-induced MMP-1 promoter activity

The effect of *F. evanescens* fucoidan on MMP-1 promoter activity was also assessed. Fucoidan significantly inhibited UVB-induced MMP-1 promoter activity by 28.4% and 49.6% at respective concentrations of 10 and 100 $\mu\text{g/ml}$ compared to UVB irradiation alone (Fig. 4).

Effect of *F. evanescens* fucoidan on UVB induced phosphorylation of MAPKs

To investigate whether fucoidan could inhibit UVB-induced activation of MAPKs, expression of phosphorylated MAPKs were evaluated by Western blotting. As shown in Fig. 5, UVB irradiation significantly induced the phosphorylation of ERK, JNK and p38. Fucoidan significantly inhibited UVB-induced ERK activation by 18.9%, 25.7%, and 39.2% at respective concentrations of 1, 10, and 100 $\mu\text{g/ml}$ compared to UVB irradiation alone ($p < 0.05$). Also, fucoidan decreased activation of JNK and p38 by 34.0% and 41.4%

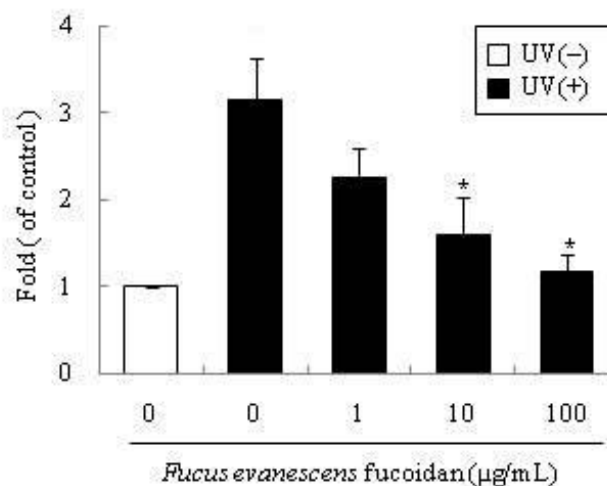


Fig. 4. Inhibition of UVB-induced MMP-1 promoter activity by *F. evanescens* fucoidan in human keratinocyte cells. After transfection, HaCaT cells were washed twice with PBS and treated with fucoidan overnight. PBS was added to quiescent cells prior to UVB exposure (15 mJ/cm^2). After UVB irradiation, cells were washed with PBS and further incubated for 24 hr. MMP-1 promoter activity determined by luminometer. Values are presented as means \pm SEM of 5 independent experiments. * $p < 0.05$ compared to UVB irradiation alone group.

at 100 $\mu\text{g/ml}$, respectively, compared to UVB irradiation alone ($p < 0.05$). Given that ERK, JNK and p38 activation is required for UVB-induced MMP-1 expression in cells, we suggest that fucoidan inhibit the expression of MMP-1 by UVB irradiation via ERK activation.

Discussion

Marine natural products provide a rich source of chemical diversity that can be used to design and develop new and potentially useful therapeutic agents that retard skin aging. Especially, several new anti-skin aging compounds have recently been isolated from brown seaweed. Brown seaweed has long been a staple of both Korean and Japanese diets and has been used in traditional Chinese medicine for over 1,000 years [26]. Senni *et al.* [31] reported that polysaccharides from brown algae stimulate dermal fibroblast proliferation and extracellular matrix deposition *in vitro* enabling the control of important parameters involved in connective tissue breakdown. Joe *et al.* [16] screened active compounds from 29 marine natural products that were capable of inhibiting MMP-1 expression in human dermal fibroblasts and identified eckol and dieckol from *Ecklonia stolonifera* as

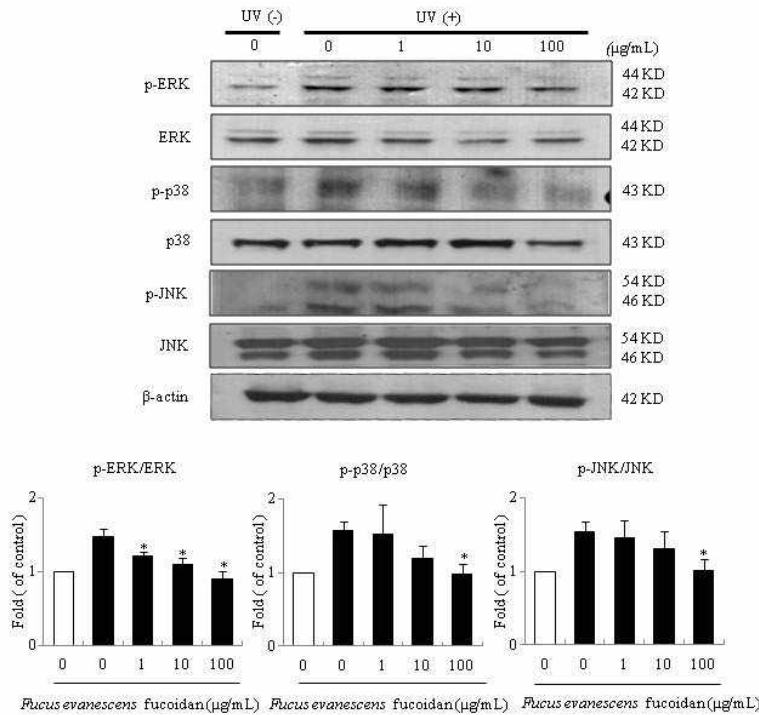


Fig. 5. Inhibition of UVB-induced phosphorylation of MAPK proteins by *F. evanescens* fucoidan in human keratinocyte cells. After treated with fucoidan for 24 hr, HaCaT cells were mock-treated or irradiated with UVB (15 mJ/cm²). The cells were washed twice with PBS and further incubated for 15 min. Phosphorylation of MAPKs was determined by Western blotting. Values are presented as means±SEM of 5 independent experiments. **p*<0.05 compared to UVB irradiation alone group.

major and active inhibitors of MMP-1 expression. The latter authors suggested that development of an agent for the prevention and treatment of skin aging was possible. We also previously reported that fucoidan inhibits UVB-induced MMP-1 expression in human skin fibroblasts at the mRNA and protein levels [28]. The results of the present study add weight to the potential use of fucoidan as a therapeutic agent to prevent and treat skin photoaging.

F. evanescens, which belongs to the order *Fucales*, is a characteristic producer of fucoidan. *F. evanescens* fucoidan has been studied for biological functions that include anticoagulant activity [19,21], antitumor activity [1], antiangiogenic and antiadhesive activities [8], and antiviral activity [22]. However, there have been no reports about the effect of *F. evanescens* fucoidan on skin aging.

Using various *in vitro* experiments, we studied the influence of *F. evanescens* fucoidan on MMP-1 expression. UVB-irradiation induced MMP-1 expression in HaCaT keratinocytes, and, as expected, fucoidan significantly inhibited UVB-induced MMP-1 mRNA and protein expression in a dose-dependent manner compared to UVB irradiation alone. Fisher *et al.* [12] have reported that the MMP-1 mRNA and protein levels are associated with MMP-1 expression. MMPs play an important role in the tissue remodeling; UVB induced MMP-1 degrades collagen type I and is the key enzyme involved in collagen breakdown in the skin [15]. The

physiological consequences of increased MMP-1 include elevated collagen degradation in the skin and the subsequent formation of wrinkles [14]. Therefore, agents that inhibit collagenase activity could have beneficial effects for maintaining healthy skin by preventing degradation of the dermal matrix.

It has been suggested that MMP-1 gene expression may be regulated in a cell-type specific manner that includes transcriptional and post-transcriptional mechanisms [3]. Also, Sun *et al.* [32] reported that the activity of MMP-1 is stringently regulated at three levels: promoter, activation of proenzyme, and inhibition of active enzyme. Murphy *et al.* [29] reported that the regulation of MMPs occurs primarily at the level of transcription activity. We confirmed that MMP-1 activation is regulated by UVB, and observed that UVB-induced MMP-1 promoter activity is inhibited by *F. evanescens* fucoidan compared to UVB irradiation alone.

MMP-1 is up-regulated via UVB-induced ERK, JNK, and p38 signaling pathways with these pathways being involved in UVB-induced MMP-1 expression [17]. We found that UVB-induced MMP-1 expression was inhibited by these substances. Therefore, we hypothesize that fucoidan may inhibit MMP-1 expression by blocking the ERK, JNK, and p38 signaling pathways. We also found that ERK activation was markedly inhibited by treatment with *F. evanescens* fucoidan. However, JNK and p38 activation were only marginally af-

ected by fucoidan. Our results demonstrate that *F. evanescens* fucoidan mainly affects inhibition of ERK activation.

Our data indicate that *F. evanescens* fucoidan may prevent UVB-induced MMP-1 expression transcriptionally, translationally, and by affecting protein function. Also, it may prevent UVB-induced MMP-1 expression by inhibiting the UVB-induced activation of the ERK signaling pathway. We suggest that *F. evanescens* fucoidan is a potential therapeutic agent that may remediate and perhaps even curtail photoaging of the skin.

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초록 : *Fucus evanescens* fucoidan의 matrix metalloproteinase-1 promoter, mRNA, 단백질 발현과 신호전달경로에 미치는 효과

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Fucoidan은 갈조류의 세포벽에 존재하는 황산화 다당류이다. 본 연구에서는 자외선 B를 인체각질형성세포에 조사하여 matrix metalloproteinase-1 (MMP-1)을 발현 시킨 후 *Fucus evanescens* fucoidan이 MMP-1 promoter, mRNA, 단백질 발현과 mitogen-activated protein kinases (MAPKs)의 인산화에 미치는 영향을 확인하고자 하였다. 자외선 B에 의해 생성된 MMP-1의 promoter activity와 mRNA, 단백질 발현은 fucoidan 10 µg/ml와 100 µg/ml를 투여하였을 때 fucoidan을 투여하지 않고 자외선만 조사한 군에 비하여 유의하게 억제되었다. 그리고 *F. evanescens* fucoidan은 extracellular signal regulated kinase (ERK)의 활성화는 현저히 억제시켰으나 c-JUN N-terminal kinase (JNK)와 p38의 활성화에 미치는 영향은 약하였다. 따라서 이 연구결과들은 *F. evanescens* fucoidan이 피부 광노화의 예방과 치료에 도움이 될 가능성을 확인할 수 있었다.