Eur J Dermatol 2009; 19 (2): 129-34

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Article accepted on 29/10/2008

Fucoidan inhibits UVB-induced MMP-1 promoter expression and down regulation of type I procollagen synthesis in human skin fibroblasts

UVB reduces type I procollagen levels and increases matrix metalloproteinases-1 (MMP-1) levels in human skin and plays a major role in the process of photoaging. We previously reported that fucoidan inhibits UVB-induced MMP-1 expression at the protein and mRNA levels in human skin fibroblasts (HS68). Yet, the effects of fucoidan on UVB-induced MMP-1 promoter activity and type I procollagen have not been investigated. In this study, we assessed the effects of fucoidan on the inhibition of MMP-1 promoter activity and on the increase of type I procollagen synthesis in human skin fibroblasts. Fucoidan treatment significantly inhibited MMP-1 promoter activity compared to UVB irradiation alone. Fucoidan treatment also increased type I procollagen mRNA and protein expression in a dose-dependent manner compared to the control. Our data indicate that fucoidan may prevent UVB-induced MMP-1 expression and inhibit down-regulation of type I procollagen synthesis. We suggest that fucoidan may be a potential therapeutic agent to prevent and treat skin photoaging.

Key words: fucoidan, matrix metalloproteinases-1, photoaging, type I procollagen, ultraviolet B, promoter activity

Aging of the skin is primarily related to a reduction in type I collagen levels, which is the principal component of skin dermis. 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 has been suggested that excessive matrix degradation by 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 [1-4], through cleavage of fibrillar collagen (type I and III in skin) at a single site within its central triple helix [5]. This evidence suggests that the expression of MMP-1 and the down-regulation of type I collagen synthesis plays a major role 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 most likely a major contributor to the phenotype of photoaged human skin [6].

The fucoidans are a family of sulphated polyfucose polysaccharides. They have attracted considerable biotechnological research interest since the discovery that they possess anti-coagulant activities similar to those of heparin [7]. They have been reported to produce antithrombotic, anti-inflammatory, anti-tumour, anti-adhesive, and anti-viral effects [8]. Recently, fucoidan was studied for potential biological activities in anti-skin aging. Using *in vitro* models of dermal wound repair, O'Leary *et al.* [9] reported that fucoidan modulates the effect of TGF-β1 on fibroblast proliferation and wound repopulation during wound repair. It has also been reported that L-fucose and fucose-rich polysaccharide preparations are efficient modulators of MMP-2 and MMP-9 activity [10], and that they increase elastic fibre surface density in rat skin and tropoelastin biosynthesis *in vitro* [11]. Percutaneous application of an L-fucose-containing preparation produced an increase of skin thickness and a densification of collagen bundles [12]. We have previously shown that fucoidan inhibits UVB-induced MMP-1 expression in human skin fibroblasts at the protein and mRNA levels [13].

In this study, we assessed the effects of fucoidan on the inhibition of MMP-1 promoter activity and the increase of type I procollagen synthesis in human skin fibroblasts.

Materials and methods

Cell culture

The normal human newborn foreskin fibroblast cell line, HS68 cell (ATCC CRL 1635), was obtained from the American Type Culture Collection (Rockville, MD). 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; Grand Island,

doi: 10.1684/ejd.2008.0611

NY). Fucoidan (Sigma; St. Louis, MO) was dissolved in distilled water. For treatment, the cells were maintained in culture media without FBS overnight, followed by treatment with fucoidan for 24 h. The cells were rinsed twice with phosphate-buffered saline (PBS), and all UVB irradiation exposures were performed under a thin layer of PBS (GIBCO; Grand Island, NY). Immediately after irradiation, the cells were incubated in serum-free fresh culture media containing fucoidan.

Ultraviolet irradiation

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 100 mJ/cm^2 dose of UVB light.

Western blotting

The cells were lysed with a 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 DTT containing 1% Triton X-100). The supernatant extracts were centrifuged at 12,000 × g for 10 min at 4 °C and used for western blot analysis. Equal amounts of protein were resolved in gradient (10%) SDS PAGE gels (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 (Calbiochem; San Diego, CA) and the type 1 procollagen antibodies (Santa Cruz Biotechnology; Santa Cruz, CA). The proteins resulting from the western blot were visualized by enhanced chemiluminescence.

RNA extraction and reverse transcriptase (RT)-polymerase chain reaction (PCR)

To assay for the MMP-1 mRNA, total RNA was isolated using the procedure of Chomczynski and Sacchi [14]. RNA concentration was quantified by UV spectrophotometer 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. The RT-PCR assay was performed to specifically quantify the mRNA level. In all of the assays, the cDNA was amplified using a standardized program (5 min denaturing steps, 30 cycles of 30 seconds at 94 °C, 30 seconds at 55 °C, and 30 seconds at 72 °C, melting point analysis in 1 °C steps, and a final cooling step) using a Gene Amp PCR 2400 (Applied Biosystem; 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′. For the type I procollagen they were forward 5′-CTC GAG GTG GAC ACC ACC CT-3′ and reverse 5′-CAG CTG GAT GGC CAC ATC GG-3′.

Plasmid constructs

We used a genomic DNA as a PCR template and primers at –2,270 bp and + 30 bp to generate a fragment containing a Sac I site at 5' end and a Hind III site at 3' end. PCR for human MMP-1 promoter was carried out using a GeneAmp® PCR System 2700 (Applied Biosystem; Foster City, CA). PCR was performed in 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 QIAquick PCR purifiction kit (Qiagen, Hilden, Germany) and then enzyme digestion by Sac I and Hind III was carried out. After the gel electrophoresis, each DNA of 2.3 kb size was extracted from gel by gel extraction kit (Qiagen, Hilden, Germany). –2,300 MMP-1 promoter was subcloned into pGEM® T easy vector according to the manufacturer's instructions. Some of the white colonies were checked to see whether they were ligated or not by Mini preparation and ligated DNA was sequenced. pGL3-basic vector was digested by Sac I restriction enzyme at 37 °C overnight. The DNA was precipitated to exchange reaction buffer. The DNA was digested by Hind III restriction enzyme at 37 °C overnight and gel electrophoresis was performed. The DNA of 4.8 kb size was extracted from gel by gel extraction kit (Qiagen, Hilden, Germany). –2,300 MMP-1 promoter subcloned into pGEM® T easy vector was digested using SacI and Hind III restriction enzyme. They were gel electrophoresed and extracted from gel. The prepared pGL3-basic vector and –2,300 MMP-1 promoter were ligated in the same way as a pGEM® T easy vector system.

Transient transfection and luciferase assay

The cells were seeded in 6 well plates at 3 × 10^5 cells/well with 2 mL of media and grown for 24 h. Transfection experiments were carried out with the Lipofectamin 2000 (Invitrogen; Carlsbad, CA) according to the manufacturer's instructions. Transfection efficiency was measured by the X-gal staining method to optimize the condition. The plasmids used were 2.5 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 1, 10, or 100 μg/mL of fucoidan in serum-free media overnight. The cells were then washed twice with PBS and irradiated with UVB at a dose of 100 mJ/cm^2. Luciferase activity was determined with a luminometer (TD 20/20, 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 ± SD and were analyzed by analysis of variance (ANOVA) followed by Duncan's test. The significance level was $p < 0.05$.

Results

Effect of fucoidan on UVB induced MMP-1 expression

The cells were treated for 24 h with various treatment concentrations of fucoidan (1, 10 or 100 μg/mL) followed by UVB irradiation (100 mJ/cm^2). The cells were further incubated for an additional 24 h. Fucoidan treatment significantly inhibited the expression of MMP-1 in a dose-dependent manner *(figure 1)*.

Effect of fucoidan on UVB induced MMP-1 mRNA expression

To study the inhibitory effects 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 *figure 2*, the result was consistent with the finding in *figure 1*. UVB-induced MMP-1 mRNA expression was significantly inhibited by the action of fucoidan; treatment of fucoidan with 1, 10 or 100 μg/mL inhibited MMP-1 expression by 70.7%, 77.2% and 81.6%, respectively, compared to UVB irradiation alone ($p < 0.05$) *(figure 2)*. Furthermore, when the cells were treated with fucoidan without UVB irradiation, the expression of MMP-1 mRNA was not changed compared to a control (data not shown).

Effect of fucoidan on MMP-1 promoter activity

Time-dependent regulation (0-24 h) of the MMP-1 promoter by UVB irradiation was examined by luciferase assay. MMP-1 promoter activity gradually increased in a time-dependent manner with maximal induction at 24 h in UVB-induced cells *(figure 3)*. Because previous studies have shown that fucoidan can inhibit the expression of MMP-1 mRNA in UVB-induced cell, the effect of fucoidan on MMP-1 promoter activity was assessed. Fucoidan inhibited UVB-induced MMP-1 promoter activity by 8.5% at 1 μg/mL, by 45.7% at 10 μg/mL, by 57.8% at 100 μg/mL, compared to UVB irradiation alone ($p < 0.05$) *(figure 4)*.

Effect of fucoidan on type I procollagen protein and mRNA expressions

We assessed the effect of fucoidan on type I procollagen synthesis. Type I procollagen protein expression was increased by 1.51-fold at 1 μg/mL, 2.08-fold at 10 μg/mL,

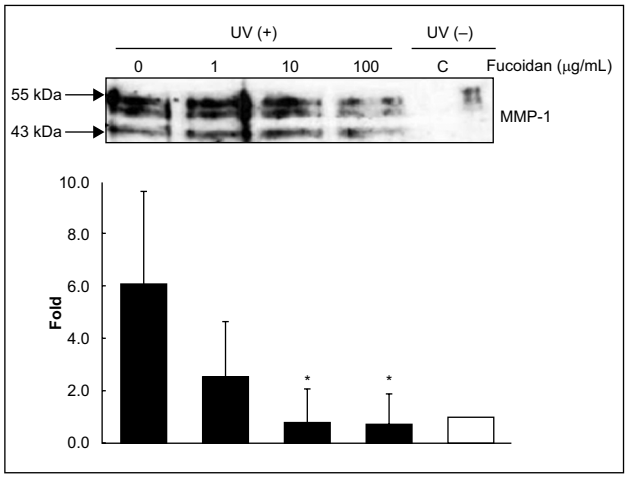

Figure 1. Inhibition of UVB-induced MMP-1 protein expression by fucoidan in human foreskin fibroblasts (HS68). After treated with fucoidan for 24 h, cells were mock-treated or irradiated with UVB (100 mJ/cm^2). The cells were washed with PBS and further incubated for 24 h. MMP-1 expression was determined in culture media by western blotting. Each bar of the lower figure shows data of MMP-1 expression quantified by densitometry. Values are presented as means ± SD of 3 independent experiments. *$p < 0.05$ compared to UVB irradiation alone group.

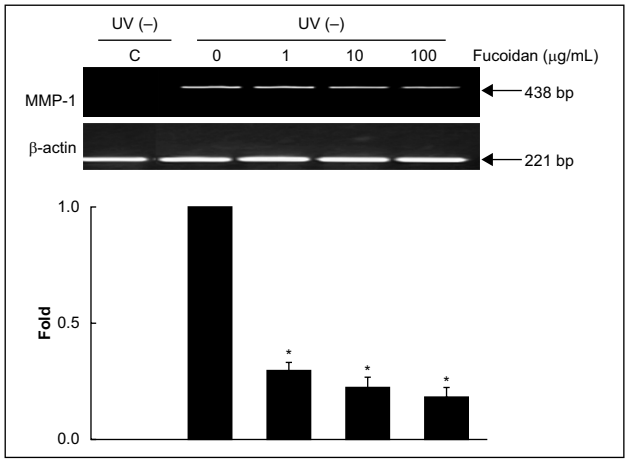

Figure 2. Effect of UVB-induced MMP-1 mRNA expression by fucoidan in human skin fibroblasts (HS68). PBS was added to quiescent cells prior to UVB exposure (100 mJ/cm^2). After UVB irradiation, the cells were washed with PBS and further incubated for 24 h. MMP-1 mRNA was determined by RT-PCR. Each bar of the lower figure shows data of MMP-1 expression quantified by densitometry. Values are presented as means ± SD of 3 independent experiments. *$p < 0.05$ compared to UVB irradiati on alone group.

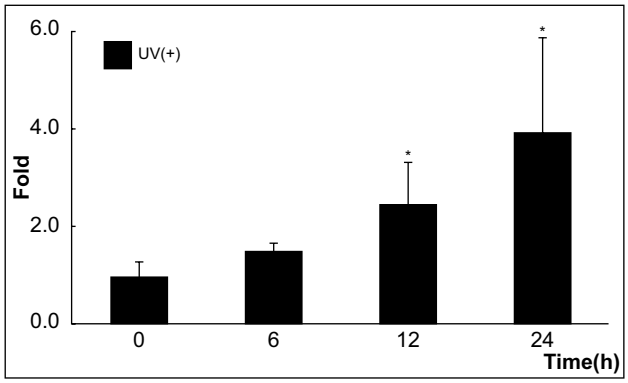

Figure 3. MMP-1 promoter activity after dose-dependent irradiation of UVB in human foreskin fibroblasts (HS68). After transfection, the cells were washed twice with PBS. PBS was added to quiescent cells prior to UVB exposure (100 mJ/cm^2). After UVB irradiation, the cells were washed with PBS and were further incubated for indicated time. MMP-1 promoter activity determined by luminometer. Values are presented as means ± SD of 3 independent experiments. *$p < 0.05$ compared to UVB irradiation alone group.

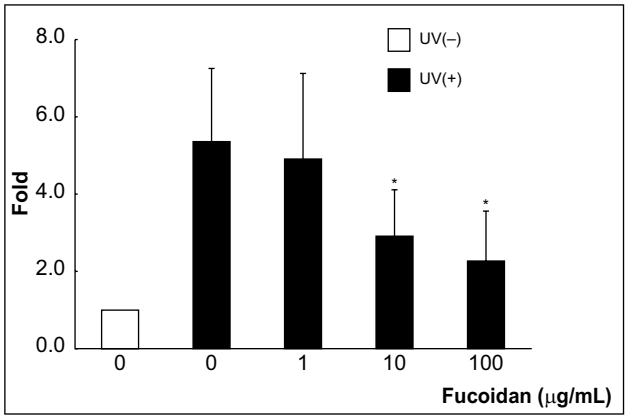

Figure 4. Inhibition of UVB-induced MMP-1 promoter activity by fucoidan in human foreskin fibroblasts (HS68). After transfection, the cells were washed twice with PBS and treated with fucoidan overnight. PBS was added to quiescent cells prior to UVB exposure (100 mJ/cm^2). After UVB irradiation, cells were washed with PBS and further incubated for 24 h. MMP-1 promoter activity determined by luminometer. Values are presented as means ± SD of 3 independent experiments. *p < 0.05 compared with the control.

and 2.26-fold at 100 μg/mL of fucoidan compared to UVB irradiation alone (p < 0.05) *(figure 5)*.

Also, to study the up-regulation effects of fucoidan on the synthesis of type I procollagen at mRNA level, RT-PCR analysis was performed. As expected, UVB-induced type I procollagen mRNA expression was increased by fucoidan. Fucoidan significantly increased type I procollagen mRNA expression in a dose-dependent manner (p < 0.05) *(figure 6)*.

Discussion

Collagen is the main component of the extracellular matrix of dermal connective tissue, and its concentration decreases with chrono- and photoaging. Once collagen is initially cleaved by MMP-1, MMP-3, and other MMPs, collagen breakdown is further promoted. The enzyme mainly responsible for collagen breakdown in skin is, MMP-1 (fibroblast collagenase), which cleaves types I, III, VII, VIII and X collagen.

Varani *et al.* [15] reported that with increasing age, MMP-1 levels rise and collagen synthesis declines for sun-protected human skin *in vivo*. Hence, the development of MMP-1 inhibitors and methods to increase synthesis of type I procollagen is considered to be a promising strategy for skin cancer therapy and photoaging. For example, some flavonoid compounds, such as naringenin, apigenin, wogonin, kaempferol, and quercetin have already been reported to regulate MMP-1 and type I procollagen expression levels [16].

Using various *in vitro* experiments, we previously reported an inhibitory effect of fucoidan on MMP-1 expression and elucidated its inhibitory pathways [13], yet the effects of fucoidan on MMP-1 transcription have not been investigated. Recently Benbow and Brinckerhoff [17] suggested that MMP gene expression may be regulated in a cell-type specific manner that includes transcrip-

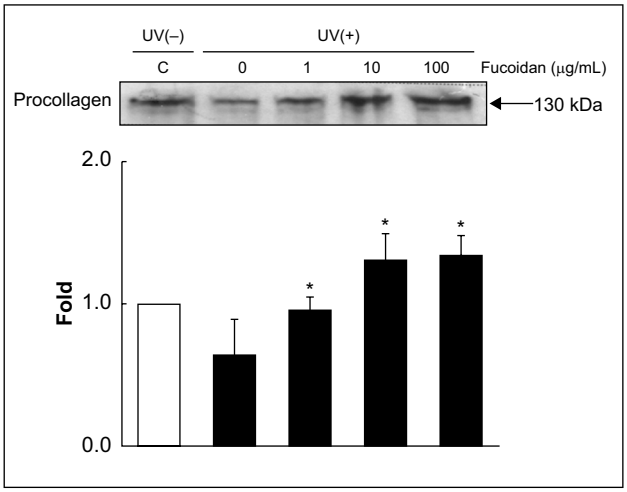

Figure 5. The effect of fucoidan on the synthesis of type I procollagen at protein level caused by UVB irradiation in human foreskin fibroblasts (HS68). After treated for 24 h with fucoidan, the cells were mock-treated or irradiated with UVB (100 mJ/cm^2). The cells were washed with PBS and further incubated for 24 h. Type I procollagen synthesis was determined in culture media by western blotting. Each bar of the lower figure shows data of type I procollagen quantified by densitometry. Values are presented as means ± SD of 3 independent experiments. *p < 0.05 compared to UVB irradiation alone group.

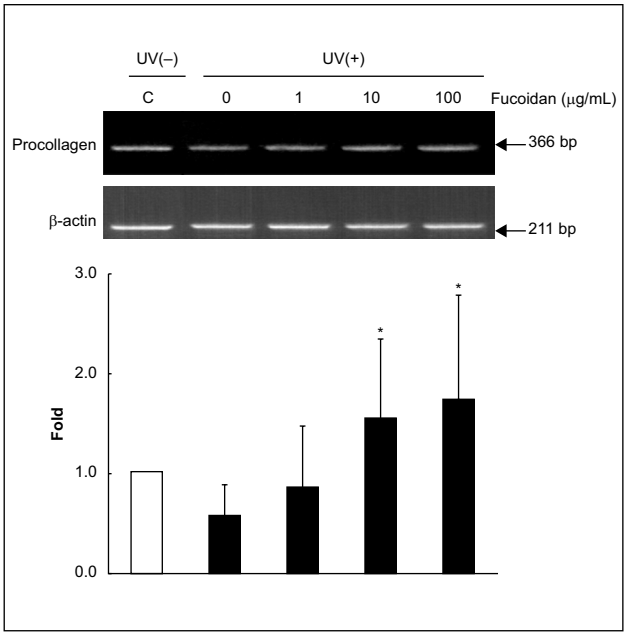

Figure 6. The effect of fucoidan on the synthesis of type I procollagen at mRNA level by UVB irradiation in human foreskin fibroblasts (HS68). After treated for 24 h with fucoidan, the cells were mock-treated or irradiated with UVB (100 mJ/cm^2). The cells were washed with PBS and further incubated for 24 h. Type I procollagen synthesis was determined by RT-PCR. Each bar of the lower figure shows data of type I procollagen quantified by densitometry. Values are presented as means ± SD of 3 independent experiments. *p < 0.05 compared to UVB irradiation alone group.

tional and post-transcriptional mechanisms. Also, Sun *et al.* [18] reported that the activity of MMP-1 was stringently regulated at three levels: the promoter, the activation of pro-enzyme, and the inhibition of active enzyme. Murphy *et al.* [19] reported that the regulation of MMPs occurred primarily at the level of transcription activity.

We found that UVB stimulated MMP-1 promoter activity in a time-dependent manner, confirmed that MMP-1 activation is regulated by UVB, and observed that UVB-induced MMP-1 promoter activity was maximally and significantly inhibited by fucoidan at 100 μg/mL *in vitro* 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 [20], and further studies have also indicated that a suppressor of AP-1 inhibits MMP-1 promoter regulation [21-24]. Therefore, additional studies are needed to identify the critical regulatory-transcriptional factors and the MMP-1 promoter regulatory regions controlled by fucoidan.

We also found that fucoidan significantly inhibited UVB-induced MMP-1 mRNA expression in a dose-dependent manner, and, as expected, the UVB-induced MMP-1 protein expression was also inhibited by fucoidan treatment compared to UVB irradiation alone.

To confirm the effect of fucoidan on type I procollagen synthesis, we performed RT-PCR and western blot analysis. Two mechanisms contribute to reduced type I procollagen synthesis: i) UV irradiation induces the transcription factor AP-1. By binding and sequestering factors that are part of a transcriptional complex required for type I procollagen transcription, AP-1 interferes with collagen production [25, 26]. Transcription factor AP-1 has also been shown to decrease collagen synthesis by blocking the effects of transforming growth factor-β (TGF-β), a major profibrotic cytokine, and sequestering one of the signaling proteins it activates both directly and indirectly [26-30]. ii) Ultraviolet irradiation also interferes with TGF-β-dependent type I procollagen gene expression by down-regulating type II TGF-β receptors, within 8 hours of irradiation, rendering the cells unresponsive to TGF-β effects [6]. Senni *et al.* [31] reported that polysaccharides were able to stimulate dermal fibroblast proliferation and extracellular matrix deposition *in vitro*, to control important parameters involved in connective tissue breakdown. Similar to their results, we observed that fucoidan treatment increased type I procollagen mRNA and protein synthesis, in a dose-dependent manner.

Our data indicate that fucoidan may prevent UVB-induced MMP-1 expression and inhibit the down-regulation of type I procollagen synthesis. We suggest that fucoidan is a potential therapeutic agent for the prevention and treatment for photoaging of the skin. Further *in vivo* studies are necessary to elucidate the anti-photoaging effects of fucoidan. ■

Acknowledgements. *This work was supported by the Korea Foundation for International Cooperation of Science & Technology (KICOS) through a grant provided by the Korean Ministry of Science & Technology (MOST) in 2006 (M60606000001-06A0600-00140). The authors have declared no conflict of interest.*

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