Biochemical and Biophysical Research Communications 450 (2014) 1333–1338

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Fucoidan/FGF-2 induces angiogenesis through JNK- and p38-mediated activation of AKT/MMP-2 signalling

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ARTICLE INFO

Article history:
Received 25 June 2014
Available online 5 July 2014

Keywords:
Fucoidan
Angiogenesis
Mitogen-activated protein kinase (MAPK)
MMP-2
AKT

ABSTRACT

Angiogenesis is an important biological process in tissue development and repair. Fucoidan has previously been shown to potentiate *in vitro* tube formation in the presence of basic fibroblast growth factor (FGF-2). However, the underlying molecular mechanism remains largely unknown. This study was designed to investigate the action of fucoidan in angiogenesis in human umbilical vein endothelial cells (HUVECs) and to explore fucoidan-signalling pathways. First, we evaluated the effect of fucoidan on cell proliferation. Matrigel-based tube formation and wound healing assays were performed to investigate angiogenesis. Matrix metalloproteinase-2 (MMP-2) mRNA expression and activity levels were analysed by reverse transcription polymerase chain reaction (RT-PCR) and zymography, respectively. Additionally, phosphorylation of mitogen-activated protein kinases (MAPKs) and protein kinase B (AKT) was detected by Western blot. The results indicate that fucoidan treatment significantly increased cell proliferation in the presence of FGF-2. Moreover, compared to the effect of FGF-2 alone, fucoidan and FGF-2 had a greater effect on tube formation and cell migration, and this effect was found to be synergistic. Furthermore, fucoidan enhanced the phosphorylation of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38, and AKT. MMP-2 activation was also significantly increased. Specific inhibitors of p38 (SB203580) and JNK (SP600125) inhibited tube formation and wound healing, while an ERK inhibitor (PD98059) did not. MMP-2 activation and AKT phosphorylation were also attenuated and associated with the suppression of p38 and JNK phosphorylation, but not with that of ERK. These results indicate that fucoidan, in the presence of FGF-2, induces angiogenesis through AKT/MMP-2 signalling by activating p38 and JNK. These findings provide basic molecular information on the effect of fucoidan on angiogenesis in the presence of FGF-2.

1. Introduction

Angiogenesis is the physiological process through which new blood vessels are formed from pre-existing vessels [1]. Angiogenesis is important in several physiological processes such as reproduction, development, and repair processes [2]. Although abnormal angiogenesis plays a role in tumour development and angioproliferative diseases, pharmacological stimulation of angiogenesis could be used to accelerate tissue regeneration [3]. Angiogenesis involves migration, proliferation, and differentiation of mature endothelial cells and is regulated by the interactions of endothelial cells with angiogenesis-stimulating factors such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF) on endothelial cells [4].

Among these molecules, FGFs belong to a family of heparin-binding polypeptide growth factors that have over 20 members, including FGF-2 [5]. FGF-2 has been shown to promote endothelial cell modifications associated with angiogenesis [6,7]. Because of their specific biological roles, FGFs can potentially be used to induce regeneration of a wide variety of tissues, including those of the skin, blood vessels, muscles, tendon/ligament, cartilage, bone, tooth, nerves, and adipose tissues [8]. Furthermore, several

Abbreviations: AKT, protein kinase B; FGF-2, fibroblast growth factor-2; HUVECs, human umbilical vein endothelial cells; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MMP-2, matrix metalloproteinase-2; MAPK, mitogen activated protein kinases; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; RT-PCR, reverse transcription polymerase chain reaction.

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http://dx.doi.org/10.1016/j.bbrc.2014.06.137
0006-291X/

studies reported that the angiogenic effect of FGF-2 was modulated by specific polysaccharides such as fucoidan [7,9,10].

Fucoidan is a high-molecular-weight sulphated polysaccharide, with a molecular structure similar to that of heparin. Fucoidan contains L-fucose and sulphate ester groups. It is primarily derived from brown algae and seaweed [11,12] and has been extensively studied because of its wide variety of biological activities, including anti-coagulant [13], anti-inflammatory [14], and anti-tumour [15] activities. In addition, fucoidan can induce angiogenesis *in vitro* by modulating the pro-angiogenic properties of heparin-binding growth factors such as FGF-2. Recent studies reported that fucoidan promotes FGF-2-induced angiogenesis in endothelial cells [7,9] by preventing proteolytic degradation of the complex formed by FGF and its receptor [16]. However, the molecular mechanism involved in fucoidan angiogenic action is poorly understood.

Therefore, this study was designed to test the effect of fucoidan on angiogenesis and explore the underlying mechanism. Our results indicate that fucoidan can enhance FGF-2-induced angiogenesis through p38- and JNK MAPK-mediated AKT/MMP-2 signalling in endothelial cells.

2. Materials and methods

2.1. Fucoidan

Fucoidan (a broad range molecular weight polysaccharide) was purchased from Haewon Biotech (Seoul, Korea). Fucoidan, which is composed of 61.5% polysaccharide and 23.5% sulphate, was extracted from the brown seaweed *Laminaria Japonica*.

2.2. Cell culture

HUVECs, purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), were maintained in DMEM (Gibco, Gaithersburg, MD, USA) supplemented with 10% foetal bovine serum (FBS) (Gibco) and antibiotics (Gibco). The cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. Cell proliferation assay

After trypsinization, HUVECs were seeded in 96-well plates at a density of 3000 cells/well. After 24 h, the medium was renewed with or without fucoidan in the presence of FGF-2 (10 ng/mL; Sigma–Aldrich, St. Louis, MO, USA). HUVECs were treated in triplicate, and the medium (with or without fucoidan and FGF-2) was renewed after 2 d of treatment. After 6 d of incubation, the cells were counted using a colorimetric assay. An MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium,) assay was performed; and the absorbance was measured at 490 nm with a microplate reader (SpectraMAX M3; Molecular Devices, Sunnyvale, CA, USA).

2.4. Matrigel tube formation assay

The plates (96-well) were prepared by adding 70 μL/well of thawed Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) to a refrigerated plate. The gel was allowed to solidify for 1 h at 37 °C. HUVECs were previously cultured in serum-free medium for 24 h. The cells were then seeded on Matrigel (3×10^4 cells/well) with or without 10 ng/mL FGF-2 and 1 μg/mL fucoidan. The cell culture was incubated at 37 °C overnight. After incubation, the cells were stained with Calcein AM and observed under a fluorescence microscope (DM IL LED Fluo, Leica, Mannheim, Germany). The total length of tube structure was measured using Image Pro Plus (Media Cybernetics, Silver Spring, MD, USA).

2.5. Cell migration assay (in vitro wound healing assay)

HUVECs (2×10^5) were seeded in 6-well plates overnight. After cellular adhesion to the plates, 1 horizontal and 1 vertical line were created by scraping the cells with a pipet tip to make a cross. The cells were then washed with phosphate buffered saline and incubated in the medium containing FGF-2 in the presence of fucoidan.

2.6. Western blot analysis

Western blot analysis was used to detect the phosphorylation of p38, JNK, ERK, and AKT. Briefly, the cells were grown in serum-free DMEM medium for 24 h and then treated with or without fucoidan. The cells were then lysed using a cell lysis buffer (iNtRon, Sungnam, Korea). The protein concentration was determined using the Bradford assay (Bio-rad, Richmond, CA, USA). Equal amounts of proteins were loaded onto and separated on 10% SDS–PAGE gels. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane (GE Healthcare, Piscataway, NJ, USA). After blocking for 1 h at room temperature in 1% bovine serum albumin (BSA), 50 mM Tris (pH 7.5), and 100 mM NaCl (TBS) containing 0.1% Tween-20, the membrane was incubated with primary antibodies, anti-phospho-p38, phospho-JNK, phospho-ERK, and phospho-AKT (Cell Signaling Technology, Beverly, MA, USA) for 4 h at room temperature. After extensive washing in TBS and 0.1% Tween-20, the horseradish peroxidase (HRP)-conjugated secondary antibodies were added, and the incubation was prolonged for 1 h at room temperature. Finally, the membrane was developed by chemiluminescence with an ECL kit (GE Healthcare) and exposed to an X-ray film.

2.7. MMP-2 mRNA expression

HUVECs were treated with fucoidan (1 μg/mL) for 24 or 48 h, and total RNA was isolated using a total RNA isolation kit (GeneAll Biotechnology Co., Seoul, Korea). To generate cDNA for amplification, 1 μg RNA was used in a two-step RT-PCR kit (iNtRon). mRNA expression levels were quantified by real-time PCR analysis. Each reaction consisted of SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR analysis was performed using the Applied Biosystems StepOnePlus™ Instrument. Reverse transcription reactions were conducted with the Maxime PCR PreMix kit (iNtRon). The primers used for amplification were as follows: forward 5′-TGA CAT CAA GGG CAT TCA GGA G-3′ and reverse 5′-TCT GAG CGA TGC CAT CAA ATA CA-3′ for MMP-2; and forward 5′-CCA CGA AAC TAC CTT CAA CTC CA-3′ and reverse 5′-GTG ATC TCC TTC TGC ATC CTG TC-3′ for β-actin.

2.8. Zymography

Gelatinase zymography was used to analyse MMP-2 levels in the cultured medium. The gelatinolytic activity of the conditioned media was visualised on SDS–PAGE gels (10% PAGE) containing 1 mg/mL gelatin (Sigma–Aldrich). After electrophoresis, the gels were soaked in 2.5% Triton X-100 (Sigma–Aldrich) for 40 min and incubated in Tris–HCl 50 mM pH 7.5, $CaCl_2$ 10 mM, and 10 mM NaCl overnight at 37 °C and stained with Coomassie brilliant blue.

2.9. Statistical analysis

All experiments were performed at least in triplicates. The values are expressed as the mean ± standard deviation (SD). A p value less than 0.05 was considered statistically significant.

3. Results

3.1. Fucoidan enhances FGF-2-induced HUVEC proliferation

To investigate the effect of fucoidan on vascular endothelial cell proliferation, we first analysed the viability of HUVECs treated with various concentrations of fucoidan (0.1–10 μg/mL) in the presence of FGF-2. Although, FGF-2 alone did not significantly affect cell proliferation, the addition of fucoidan (0.1–1 μg/mL) significantly increased cell proliferation. In particular, a 10 μg/mL fucoidan treatment induced a 200% increase in HUVEC proliferation (Fig. 1A). These results indicate that fucoidan induced HUVEC proliferation in a dose-dependent manner, in the presence of FGF-2 *in vitro*.

3.2. Fucoidan enhances FGF-2-induced angiogenic activity

To evaluate the angiogenic activity of fucoidan, tube formation and wound healing assays were performed. First, the effect of fucoidan on tube formation was tested in the Matrigel tube formation assay after treatment with FGF-2 and/or fucoidan. The endothelial cell capillary tube and network formation were slightly increased by FGF-2. Fucoidan alone did not significantly affect tube formation. However, tube formation increased (about 4-fold) to a greater extent in cells treated with a combination of fucoidan and FGF-2 when compared to the control cells (Fig. 1B and C). Furthermore, FGF-2/fucoidan strongly promoted HUVEC migration ability induced by FGF-2 (Fig. 1D). These results indicate that fucoidan also enhances FGF-2-induced endothelial cell migration.

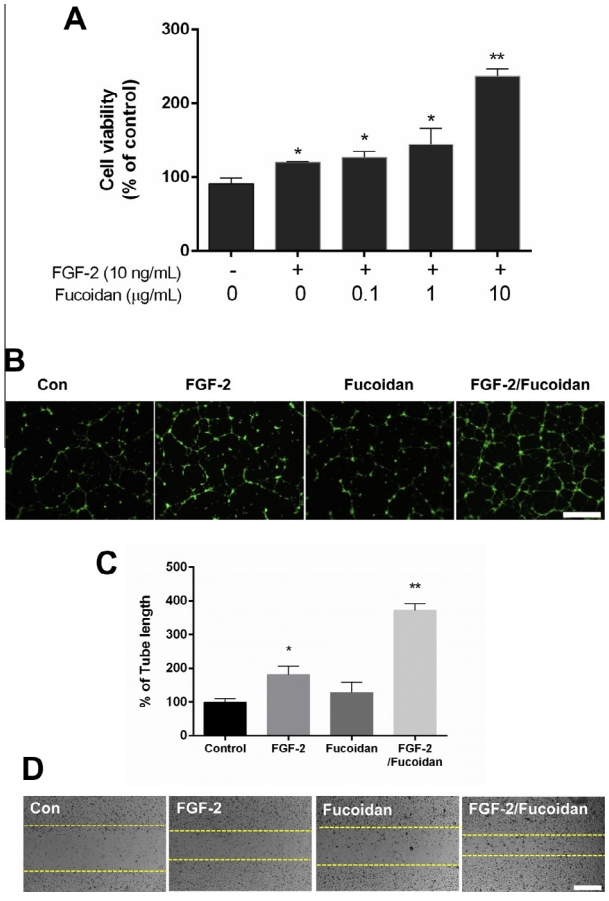

Fig. 1. Effect of fucoidan on FGF-2-induced HUVEC proliferation and angiogenesis. HUVECs were treated with FGF-2 (10 ng/mL) and various concentrations of fucoidan (0.1–10 μg/mL). HUVEC proliferation was measured after 6 d of incubation. (A) Fucoidan exhibited a synergistic effect with FGF-2 on cell proliferation in a dose-dependent manner. Angiogenic activity of fucoidan was determined by tube formation and wound healing assays. Tube formation photographs (B) and calculated total length (C) show that vascular tube formation is strongly enhanced by FGF-2/fucoidan treatment. Furthermore, wound healing assay (D) performed *in vitro* revealed that FGF-2-induced cell migration was also enhanced by fucoidan treatment. Data are presented as the mean ± SD of 3 independent experiments. Significance was defined as $^{*}p < 0.05$ or $^{**}p < 0.01$ when compared with control.

3.3. Effect of fucoidan on MAPK phosphorylation

We explored the involvement of MAPKs in fucoidan-enhanced angiogenic activity, induced by FGF-2. Fig. 2A shows that in cells treated with FGF-2 only, ERK, JNK, and p38 phosphorylation increased approximately 4 h after treatment. Furthermore, the phosphorylation of these MAPKs was also induced by fucoidan alone, but the phosphorylation was observed later (24 h after treatment). However, all MAPKs were strongly activated 2 h after the FGF-2/fucoidan treatment. The results indicate that FGF-2-induced MAPK activation was accelerated by fucoidan.

3.4. Effect of fucoidan on AKT/MMP-2 signalling

To investigate whether FGF-2/fucoidan-induced angiogenic activity was associated with AKT signalling, AKT phosphorylation level, MMP-2 mRNA expression, and MMP-2 activation were analysed by Western blot, RT-PCR, and zymography, respectively. AKT phosphorylation level was strongly increased after 4 h of treatment with FGF-2 alone. However, FGF-2/fucoidan treatment resulted in a strong increase in AKT phosphorylation after 2 h. (Fig. 2B). Furthermore, MMP-2 mRNA showed weak expression after 48 h of FGF-2 treatment alone, but was found to be strongly expressed after 24 h of FGF-2/fucoidan treatment (Fig. 2C). The zymography results also showed that the active form of MMP-2 increased with FGF-2/fucoidan treatment (Fig. 2D).

3.5. Effect of MAPK inhibitors on angiogenic activity and AKT/MMP-2 signalling

Fig. 2 shows that FGF-2/fucoidan treatment induced ERK, JNK, and p38 phosphorylation. We tested whether the angiogenic effect of fucoidan was mediated via MAPKs. Matrigel tube formation and wound healing assays were performed using cells pre-treated with specific MAPK inhibitors. As shown in Fig. 3, FGF-2/fucoidan-induced tube formation was significantly inhibited by SB203580 and SP600125, but not by PD98059 (Fig. 3A and B). Additionally, a similar trend was observed in cell migration using the wound healing assay (Fig. 3C). Western blot analysis showed that FGF-2/fucoidan treatment-induced increase in AKT phosphorylation was inhibited by pre-treatment with SB203580 and SP600125. Furthermore, MMP-2 activity was inhibited by SB203580 and SP600125, but not by PD98059 (Fig. 4).

4. Discussion

Angiogenesis plays an important role in organogenesis and development. Several studies reported that fucoidan enhanced angiogenic activity [7,9]. However, the underlying molecular mechanism has not yet been reported. In this study, we focused on the angiogenic activity of fucoidan and the associated signalling pathways such as those involving MAPKs, AKT, and MMP-2. We found that fucoidan promotes proliferation and angiogenesis, at least through the p38- and JNK-signalling pathways in HUVECs. Our data also show that fucoidan enhanced FGF-2-induced angiogenic activity through AKT and MMP-2 signalling.

We first investigated the effect of fucoidan on cell proliferation and observed that fucoidan enhanced the growth rate of endothe-

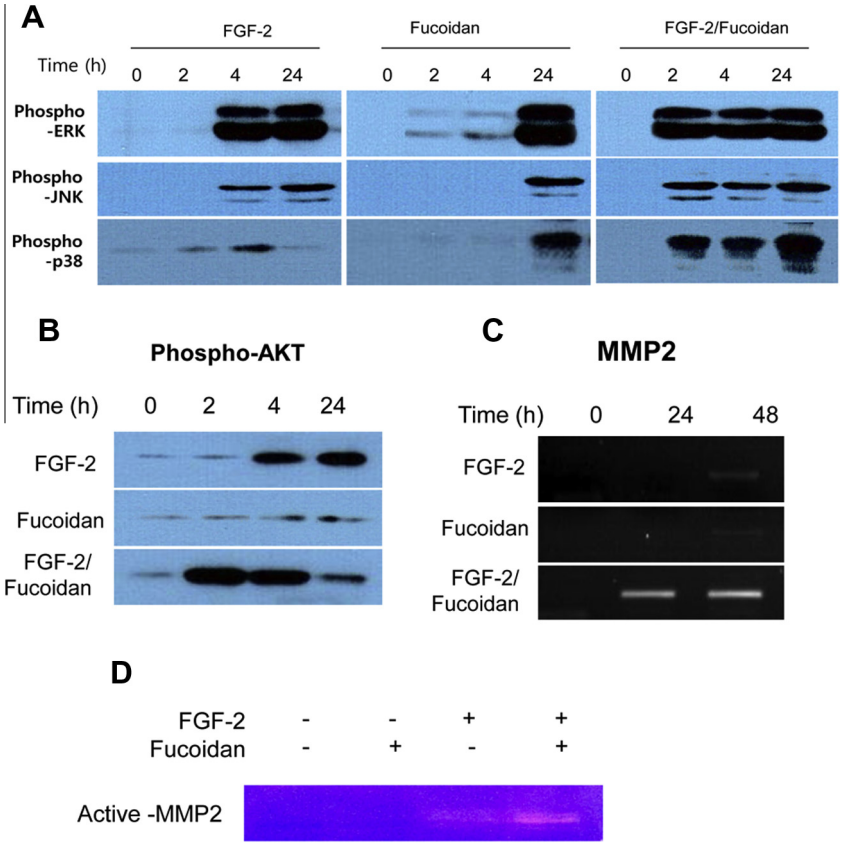

Fig. 2. Effect of fucoidan on the phosphorylation of members of the MAPK family and AKT/MMP-2 signalling in HUVECs. Effect of fucoidan on MAPK activation. Cells were treated with FGF-2 and/or fucoidan, and Western blot was performed. FGF-2 induced phosphorylation of ERK, JNK, and p38. Furthermore, FGF-2/fucoidan co-treatment accelerated MAPK activation (A). To evaluate effects of fucoidan on AKT/MMP-2 signalling, HUVECs were treated with FGF-2 and/or fucoidan. AKT phosphorylation was analysed by Western blot (B), MMP-2 mRNA expression was analysed by RT-PCR (C), and MMP-2 activation was analysed by zymography (D). AKT phosphorylation level increased with FGF-2 treatment. FGF-2/fucoidan co-treatment further increased the AKT phosphorylation level. Moreover, FGF-2/fucoidan co-treatment increased MMP-2 mRNA expression and activation.

lial cells in the presence of FGF-2 in a dose-dependent manner. Our results are in agreement with those of a previous study [17], which showed that HUVEC proliferation plateaus at specific concentrations of fucoidan. In contrast, Soeda et al. [18] reported that fucoidan had no effect on cell proliferation, and Lui et al. [19] reported that fucoidan inhibited cell proliferation. To determine whether fucoidan promotes angiogenesis, we examined tube formation and cell migration by the Matrigel tube formation assay and the wound healing assay, respectively. One study suggests that a low molecular weight fraction of fucoidan inhibited angiogenesis [17]. Additionally, a previous study reported that a high molecular weight fraction of fucoidan had no effect on FGF-2-induced tube formation at a concentration of 10 μg/mL, whereas it inhibited FGF-2-induced tube formation at a concentration of 100 μg/mL in HUVECs [18]. In contrast, Matou et al. reported that 20-kDa fucoidan enhances FGF-2–induced angiogenesis. In particular, Matou et al. used fucoidan at a concentration range of 0.1–10 μg/mL in the presence of FGF-2 (5 ng/mL) and showed that fucoidan promoted FGF-2-induced tubular structure formation in a dose-dependent manner [17]. In this study, fucoidan was found to enhance angiogenesis at a concentration of 1 μg/mL, which is consistent with the findings of Fukahori et al. [15]. These studies indicate that the molecular size of fucoidan relative to its concentration influences its effect on angiogenesis. Discrepancies in these results might be due to the difference in seaweed species and/or extraction methods used as well as the molecular weight of fucoidan and the sulphate content [20,21].

MAPK signalling is critical for the growth, survival, and migration of cells [22]. Hence, MAPK signalling was evaluated to gain further insight into the mechanisms by which fucoidan enhances FGF-2-induced angiogenesis. FGF is known to activate multiple downstream signalling pathways, including the MAPK signalling pathways, in various cell types [23–25]. Kaikai et al. reported that FGF-2 stimulation could induce phosphorylation of JNK/SAPK, ERK1/2, and p38, thereby inducing cell proliferation, migration, and tube formation in HUVECs [26].

Moreover, we were interested in elucidating the intracellular signalling pathways in FGF-2-mediated angiogenesis. In the present study, we observed that fucoidan/FGF-2 co-treatment induced phosphorylation of p38, JNK, and ERK. Furthermore, the response time was lesser in the fucoidan/FGF-2-treated cells than in the FGF-2-treated cells. Previous studies demonstrated the crucial roles of ERK and p38 in FGF-2 signalling [27–30], and FGF-2 could also trigger the phosphorylation of JNK in HUVECs [26]. Therefore, we tested whether angiogenesis was enhanced after treatment with specific MAPK inhibitors. Our results show that pre-treatment with specific p38 and JNK inhibitors, but not ERK inhibitors, inhibited fucoidan-enhanced angiogenesis. These data indicate that fucoidan enhances FGF-2-induced angiogenesis through the p38 and JNK pathways, but not through the ERK pathway.

AKT is a central signalling molecule, which regulates cell survival and angiogenesis in endothelial cells [31]. Furthermore, the MMP family, which modulates extracellular matrix degradation, plays several roles in angiogenesis. In particular, MMP-2 plays a key role in endothelial cell migration and matrix remodelling during angiogenesis [32]. We examined whether MAPK inhibitors could also inhibit fucoidan-induced AKT phosphorylation and MMP-2 activity. We found that the level of AKT phosphorylation

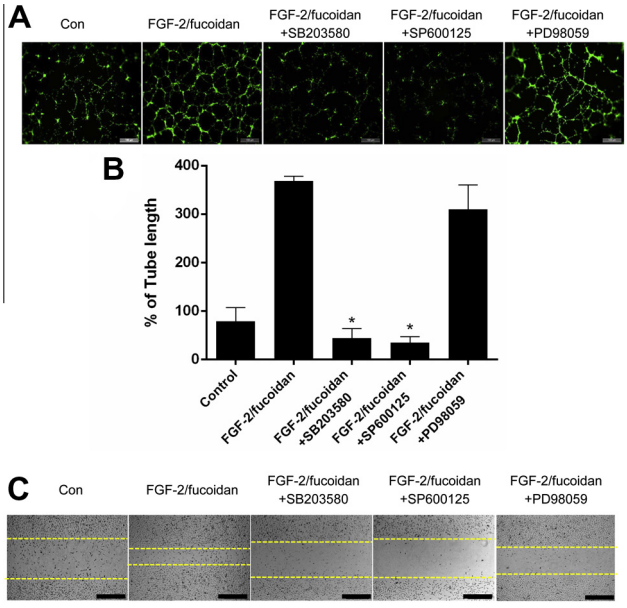

Fig. 3. Effects of MAPK inhibitor on FGF-2/fucoidan–induced HUVEC tube formation and cell migration. HUVECs were pre-treated with SB203580 (10 μM), SP600125 (10 μM), and PD98059 (20 μM) for 30 min and cultured in the presence of FGF-2 and fucoidan. After 24 h of incubation, Matrigel tube formation was evaluated. Tube formation photographs (A) and calculated total length (B) showed that FGF-2/fucoidan-induced vascular tube formation was inhibited by SB203580 and SP600125, but not by PD98059. Furthermore, the wound healing assay (C) showed that FGF-2/fucoidan-induced cell migration was inhibited by SB203580 and SP600125, but not by PD98059. Data shown are the mean ± SD. Significance was defined as $^{**}p < 0.05$ when compared with FGF-2/fucoidan treated group.

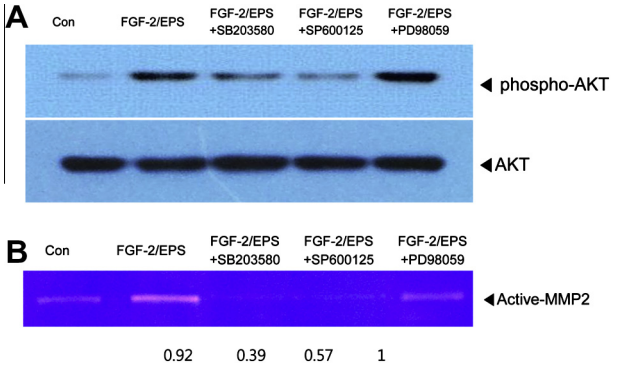

Fig. 4. Effects of MAPK inhibitor on AKT phosphorylation and MMP-2 activation. HUVECs were pre-treated with SB203580 (10 μM), SP600125 (10 μM), and PD98059 (20 μM) for 30 min and cultured in the presence of FGF-2/fucoidan. After 2 h of incubation, AKT phosphorylation was evaluated by Western blot (A). After 24 h of incubation, MMP-2 activation was determined by zymography (B). AKT phosphorylation in cells treated with FGF-2/fucoidan was inhibited by SB203580 and SP600125, but not by PD98059. Furthermore, MMP-2 activation was also inhibited by SB203580 and SP600125, but not by PD98059.

increased with fucoidan treatment. Additionally, AKT phosphorylation was inhibited by p38 and JNK inhibitors.

According to a previous report, fucoidan induces production of cytokines such as IL-6, IL-1β, VEGF-A, and FGF-2. Growth factors, including VEGF-A, FGF-2, and angiogenin, induce cytokine production and indirectly induce MMP-2 activation [33]. Notably, Hlawaty et al. [34] reported that low molecular weight fucoidan (LMWF) significantly decreased the activity of MMP-2, but increased MMP-2 mRNA expression in HUVECs. In contrast, our results show that both MMP-2 mRNA expression and activity was increased by fucoidan treatment. Although we have not been able to elucidate the reason for this in the present study, we hypothesise that the difference in the activity and mRNA expression of the enzyme is due to the molecular weight and physicochemical parameters [20,21]. Our results show that MMP-2 expression and activity increased with fucoidan treatment. Additionally, p38 and JNK inhibitors inhibited fucoidan-induced MMP-2 mRNA expression and activity. Although it remains unclear whether fucoidan directly or indirectly regulates MMP-2, these results suggest that p38 and JNK activation mediates AKT and MMP-2 signalling.

In conclusion, this study shows that fucoidan acts synergistically with FGF-2 in promoting endothelial cell proliferation and angiogenesis. We showed that fucoidan enhanced angiogenesis by AKT and MMP-2 signalling via the activation of the p38 and JNK signalling pathways. These findings provide experimental evidence demonstrating that fucoidan could be beneficial in clinical situations where active angiogenesis is required, such as wound repair.

Acknowledgments

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2013-0731).

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