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Fucoidan inhibits activation and receptor binding of transforming growth factor-β1

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ABSTRACT

Fucoidan, a sulfated, fucose-rich polysaccharide isolated from marine brown algae, has antifibrotic effects. We investigated the biologic effects of interactions of fucoidan with transforming growth factor-β1 (TGF-β1) and latent TGF-β1 (LTGF-β1). TGF-β1 bound to fucoidan was unable to interact with its receptor. In agreement with this, fucoidan attenuated the cellular effect of TGF-β1 as measured by phosphorylation of Smad2. Binding of fucoidan rendered LTGF-β1 resistant to activation as follows. Fucoidan inhibited furin-like proprotein convertase-mediated activation of platelet LTGF-β1 without suppression of the enzyme. In addition, acid- or heat-activation of small recombinant LTGF-β1 and acid-activation of large LTGF-β1 in cultured cell supernatant were also inhibited by fucoidan. Fucoidan is a mixture of polysaccharides of different sizes. As molecular weight of fucoidan increases, it had more inhibitory effects on TGF-β1 and LTGF-β1. In conclusion, inhibitions of LTGF-β1 activation and TGF-β1 receptor binding by fucoidan may in part account for its antifibrotic effects.

1. Introduction

Transforming growth factor-β1 (TGF-β1) is the representative of fibrogenic cytokines [1]. It is secreted from cells as a small latent TGF-β1 (LTGF-β1) complex, in which TGF-β1 is noncovalently bound to latency associated peptide (LAP), or more commonly as a large LTGF-β1 complex, in which latent TGF-β binding protein is disulfide-linked to LAP of a small LTGF-β1 complex [2]. LTGF-β1 itself does not bind to TGF-β receptor and thus has no biologic activity of TGF-β1. To be active, TGF-β1 has to be cleaved from LAP of the latent complex. After dissociation, TGF-β1 interacts with type II TGF-β receptor on the cell surface, which in turn recruits and activates type I TGF-β receptor [3]. Smad2 and Smad3 are phosphorylated at the carboxyl-terminal serines by the activated type I TGF-β receptor and form heteromeric complexes with Smad4. Thereafter, Smad2/3/4 complex translocates into the nucleus and binds to a specific sequence in DNA, which in turn results in transcription of the target genes, including fibronectin and collagen [3].

The process of liberation of TGF-β1 from LTGF-β1 complex *in vivo* is still poorly understood, but it may occur by enzymes including plasmin [4], matrix metalloproteinase [5] and furin-like proprotein convertase [6] or by conformational changes of LAP [7,8]. *In vitro*, LTGF-β1 can be activated by transient acidification which dissociates TGF-β1 from LAP by disrupting hydrogen bond and other noncovalent bonds through changes in ionic strength [9]. LTGF-β1 is also activated by heat treatment [10].

Fucoidan is a sulfated, fucose-rich polysaccharide abundant in marine brown algae [11]. Sulfated polysaccharides may bind to proteins. Clusters of positively charged basic amino acids on proteins form ion pairs with spatially defined negatively charged sulfo or carboxyl groups on the sulfated polysaccharides [12]. Through the interactions with growth factors and complements, fucoidan has diverse biologic effects such as anti-inflammatory and anti-tumor effects, and was suggested to be a possible therapeutic agent [13]. Animal studies have shown that fucoidan has antifibrotic effects as well, preventing hepatic fibrosis and reducing the size of myocardial infarct scar [14,15].

Fucoidan binds to TGF-β1 and it was suggested to potentiate TGF-β1 activity [16]. However, there was also a study suggesting that fucoidan may abrogate the cellular effect of TGF-β1 [17]. Thus, the effect of fucoidan on the activity of TGF-β1 remains to be further clarified.

In the present study, we investigated whether TGF-β1 bound to fucoidan binds normally to its receptor and has biological activity. In addition, we evaluated whether fucoidan binds to LTGF-β1 and modulates its activation.

Abbreviations: TGF-β1, transforming growth factor-β1; LTGF-β1, latent TGF-β1; MW, molecular weight; FF7, fucoidan fraction 7.

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2. Materials and methods

2.1. Materials

Recombinant human TGF-β1 (r-TGF-β1) and recombinant human small LTGF-β1 were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). ^{121}I-TGF-β1 was obtained from PerkinElmer Life Sciences, Inc. (Boston, MA, USA). Two commercial preparations of fucoidan were used. Fucoidan from Sigma–Aldrich Co. (St. Louis, MO, USA) was a crude fucoidan prepared from the marine brown algae *Fucus vesiculosus*. It is a mixture of fucoidan of different sizes, from thousands to over 680,000 in molecular weight (MW) [18]. Another fucoidan, named as fucoidan fraction 7 (FF7), was obtained from Calbiochem (San Diego, CA, USA). It is a purified fraction of fucoidan isolated from *F. vesiculosus*. Dec-RVKR-CMK, hexa-D-arginine (furin inhibitors) and Pyr-Arg-Thr-Lys-Arg-AMC (furin substrate) were purchased from Calbiochem. Antibodies to human Smad2/3 and phospho-Smad2 (Ser465/467) were from Cell Signaling Technology (Denvers, MA, USA).

2.2. Cell culture and collection of conditioned medium

HK-2 cells are immortalized human renal proximal tubular epithelial cells obtained from the American Type Culture Collection (Manassas, VA, USA). Growth-arrested HK-2 cells were treated with 5 ng/ml of interleukin-1β in serum-free M199 medium (Life Technologies, Paisley, UK) to obtain high concentration of LTGF-β1. After incubation for 72 h, the conditioned medium was collected and centrifuged to remove cell debris, aliquoted, and stored at −70 °C for subsequent activation studies.

2.3. Preparation of platelet lysate

Blood samples were obtained from healthy adult volunteers after informed consent was obtained. Peripheral venous blood was drawn by venipuncture of the antecubital vein, and collected in the EDTA tube. Platelet-rich plasma was centrifuged at 6000*g* for 5 min, resulting in platelet pellet. The pellet was washed with M199 media to remove EDTA, and resuspended in 1/3 original volume of M199 media/0.2% BSA. To induce hypotonic lysis, the platelet solution was mixed with equal amount of MilliQ water and placed on ice for 30 min. Thereafter, the platelet solution was centrifuged at 6000*g* for 5 min at 4 °C to eliminate lysed platelets, and the supernatant was obtained as platelet lysate.

2.4. Activation of platelet LTGF-β1

Aliquots of the platelet lysate placed on ice were mixed with different concentrations of fucoidan, 100 μM of Dec-RVKR-CMK or 100 μM of hexa-D-arginine and incubated on ice for 10 min. Thereafter, samples were placed at room temperature for 1 h to allow enzymatic activation of LTGF-β1. After incubation, the samples were subjected to enzyme-linked immunosorbent assay (ELISA) to measure active form of TGF-β1.

2.5. TGF-β1 ELISA

The binding of TGF-β1 to its receptor was measured by ELISA kit (Quantikine® Human TGF-β1 Immunoassay, R&D Systems, Inc.), in which the wells are coated with type II TGF-β receptor. The level of active TGF-β1 was measured by another sandwich ELISA kit

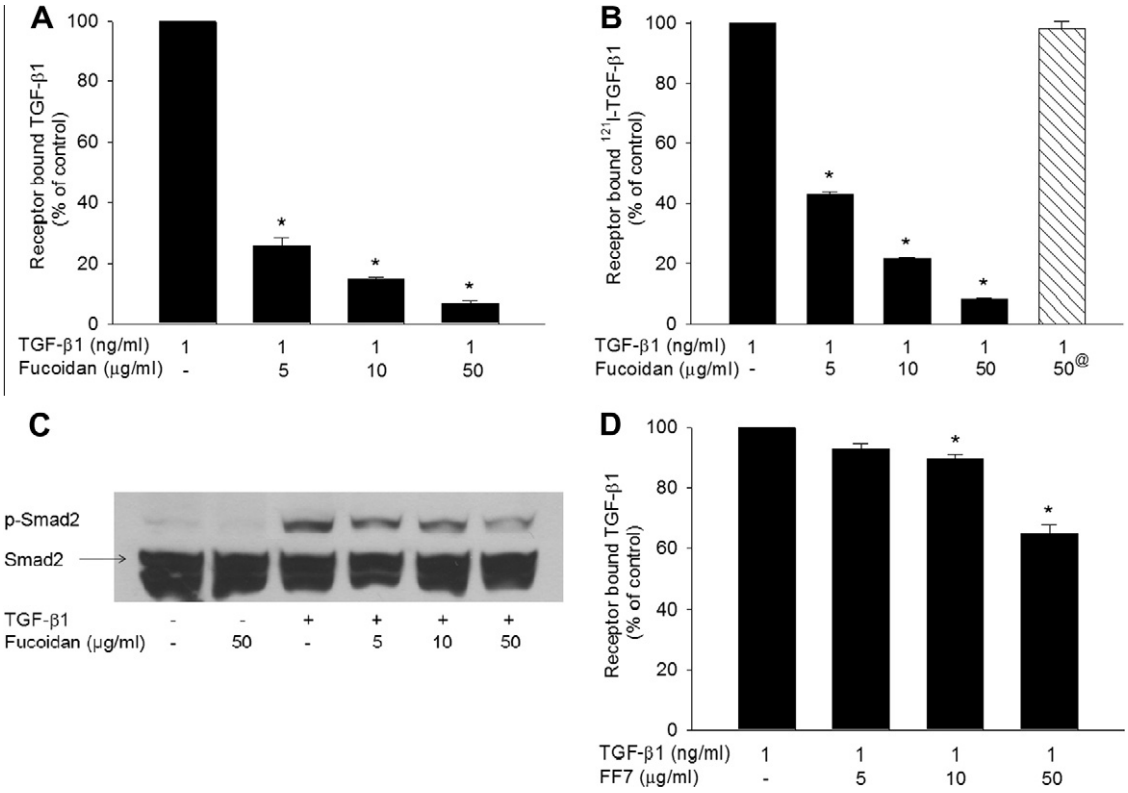

Fig. 1. Binding of fucoidan renders TGF-β1 unable to interact with its receptor. (A and D) r-TGF-β1 dissolved in PBS/0.1% BSA was mixed with different concentrations of fucoidan (A) or FF7 (D). The mixture was incubated in the wells coated with type II TGF-β receptor, and the amount of r-TGF-β1 bound to its receptor was measured by ELISA (*$p < 0.05$ compared with r-TGF-β1 without fucoidan; $n = 6$). (B) ^{121}I-TGF-β1 mixed with fucoidan was incubated for 2 h in the wells coated with type II TGF-β receptor. After washing with PBS, radioactivity of each well was measured by gamma counter. Another well@ was preincubated with fucoidan for 3 h and washed with PBS and then incubated with ^{121}I-TGF-β1 (*$p < 0.05$ compared with ^{121}I-TGF-β1 without fucoidan; $n = 6$). (C) HK-2 cells were incubated with r-TGF-β1 (1 ng/ml) mixed with or without fucoidan under serum-free condition. After 30 min incubation, whole cell lysates were obtained and subjected to immunoblotting with an anti-phospho-Smad2 antibody. The result shown is representative of three independent experiments.

(DuoSet ELISA development system, R&D Systems, Inc.), in which the wells are coated with mouse anti-TGF-β1 monoclonal antibody. Acid activation of LTGF-β1 was performed by incubation of the samples (200 μl) with 1 N HCl (40 μl) for 10 min, followed by neutralization with 1.2 N NaOH/0.5 M HEPES (40 μl). Heat activation was performed by incubating the samples for 10 min at 80 °C.

2.6. Assay of ^{121}I-TGF-β1 binding to type II TGF-β receptor

To evaluate the effects of fucoidan on the binding of ^{121}I-TGF-β1 to type II TGF-β receptor, ELISA well modules coated with type II TGF-β receptor were used. At first, each well of the module was separated by breaking the connecting portion between the wells. And then, samples containing ^{121}I-TGF-β1 were incubated in the wells, and washed out. Thereafter, each well was put in the glass tube and the radioactivity was counted by the gamma counter.

2.7. Western blot analysis

Growth-arrested HK-2 cells were incubated with TGF-β1 mixed with different concentrations of fucoidan for 30 min. Cell lysates were obtained and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 10% gel and transferred to an Immobilon-P membrane (Millipore, Bedford, MA, USA). The membrane was probed with a rabbit antibody to human phospho-Smad2. Bands were visualized using horseradish peroxidase conjugated anti-rabbit IgG (Santa Cruz Biotechnology) and the enhanced chemiluminescence agent (Amersham International).

2.8. Furin-like proprotein convertase activity assay

Furin-like proprotein convertase activity was assayed in black 96-well plates using the fluorogenic substrate, Pyr-Arg-Thr-Lys-Arg-AMC, for 2 h at room temperature. Enzyme activity was determined as the release of the fluorescent AMC moiety within the assay period, measured by SPECTRmax© GEMINI XS system (Molecular Devices, Sunnyvale, CA, USA).

2.9. Fractionation of crude fucoidan and measurement of fucoidan concentration

Fucoidan is a mixture of sulfated polysaccharides with different MW. It was divided into three fractions as a high, middle and low MW using filter devices as follows. Crude fucoidan dissolved in distilled water was filtered through an ultracentrifugal filter device of MW cutoff of 100,000 Da (Amicon Utra-4 100,000 MWCO, Millipore Corp., Bedfore, MA, USA) by centrifugation at 1500g for 20 min. The fraction remained unfiltered was designated as a high MW fucoidan. The filtered fraction was further filtered through an ultracentrifugal filter device of MW cutoff of 30,000 Da (Amicon Utra-4 30,000 MWCO, Millipore Corp.) by centrifugation at 1500g for 20 min. The filtered fraction was designated as a low MW fucoidan. The fraction remained unfiltered was designated as a middle MW fucoidan. The concentration of fractionated fucoidan was measured by anthrone method [19].

2.10. Statistical analysis

Data are presented as the mean ± SE. Student's *t*-test was used to compare two values, and an analysis of variance followed by

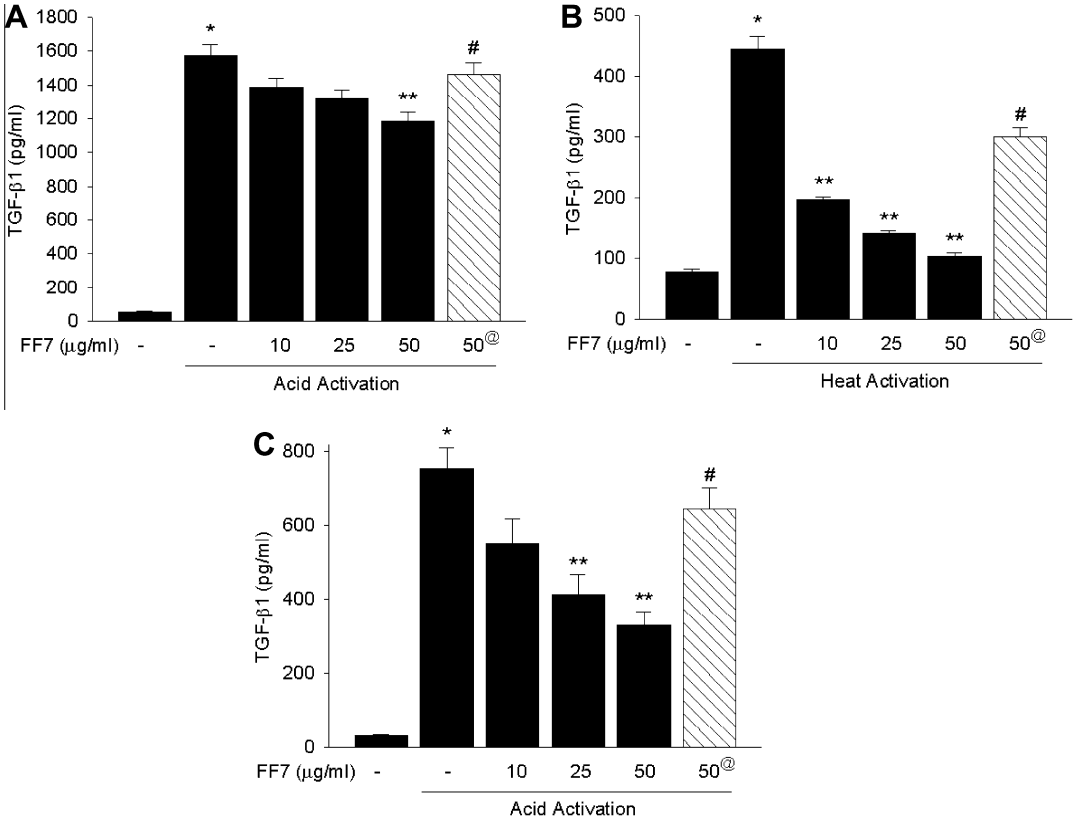

Fig. 2. FF7 inhibits acid- or heat-activation of LTGF-β1. (A and B) Recombinant LTGF-β1 was mixed with different concentrations of FF7 before acid- (A) or heat-activation (B), and activated TGF-β1 was measured by ELISA. In another sample@, recombinant LTGF-β1 was subjected to acid- (A), or heat-activation (B) and thereafter mixed with 50 μg/ml of FF7 to estimate the decrease in TGF-β1 through interaction with activated TGF-β1. (C) HK-2 cell culture supernatant was treated as with A. (*$p < 0.05$ compared with pre-activation; **$p < 0.05$ compared with activation without FF7; #$p < 0.05$ compared with 50 μg/ml of FF7 mixed before activation; $n = 6$.)

Scheffe's multiple-comparison test was used to compare mean values among three or more groups. A p-value of <0.05 was considered statistically significant.

3. Results

3.1. Fucoidan inhibits the receptor binding of TGF-β1

r-TGF-β1 was mixed with different concentrations of fucoidan and incubated in the wells coated with type II TGF-β receptor, and TGF-β1 was measured by ELISA. As shown in Fig. 1A, fucoidan dose-dependently decreased the measured TGF-β1. Similar results were also obtained in ELISA using the wells coated with mouse anti-TGF-β1 monoclonal antibody ($n = 8$, 4.5 ± 0.1% of control at 50 μg/ml of fucoidan).

To determine whether fucoidan inhibits binding of TGF-β1 to its receptor, ^{121}I-TGF-β1 was mixed with different concentrations of fucoidan and incubated in the wells coated with type II TGF-β receptor. After 3 h of incubation, the wells were washed with PBS, and the receptor-bound ^{121}I-TGF-β1 was measured by counting the radioactivity of each well in the gamma counter. As shown in Fig. 1B, the radioactivity was decreased by fucoidan in a dose-dependent manner. Fucoidan may inhibit binding of TGF-β1 to its receptor by interaction either with TGF-β1 or with TGF-β1 receptor. To differentiate it, we included another well, which was preincubated with fucoidan (50 μg/ml) for 3 h and washed with PBS before incubation with ^{121}I-TGF-β1. Pretreatment of the well with fucoidan did not have an effect on the binding of ^{121}I-TGF-β1 to its receptor, implying that fucoidan does not interact with the receptor.

In another experiment, HK-2 cells were incubated with r-TGF-β1 mixed with or without fucoidan, and the activation of Smad2 was assessed in the immunoblot of whole cell lysates using an anti-phospho-Smad2 (Ser465/467) antibody. Consistent with the above findings, fucoidan attenuated the effect of TGF-β1 (Fig. 1C).

FF7 also inhibited the binding of TGF-β1 to its receptor, but the magnitude of inhibition was much small, as compared with that by crude fucoidan (Fig. 1D).

3.2. Fucoidan inhibits acid- or heat-activation of LTGF-β1

Next, we evaluated whether fucoidan interacts with LTGF-β1 and thereby modulates its activation. As FF7 interacts with active TGF-β1 released from LTGF-β1 and partly inhibits its receptor binding, FF7 was added before or after activation procedure and the difference of the measured TGF-β1s was regarded as the portion of inhibited activation by FF7.

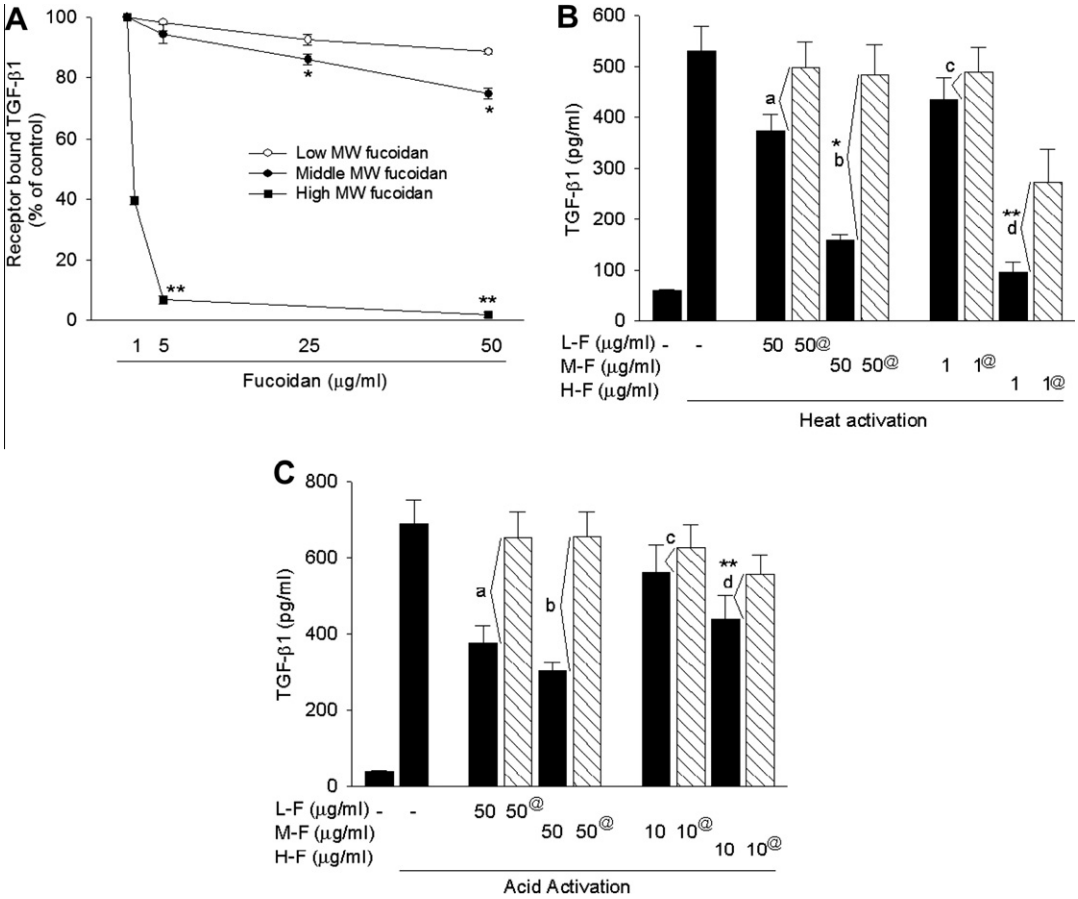

Fig. 3. The strengths of interactions of fucoidan with active and latent TGF-β1 depend on its molecular weight. Crude fucoidan was fractionated according to molecular weights; low MW (L-F), middle MW (M-F), and high MW fucoidan (H-F). (A) r-TGF-β1 dissolved in PBS/0.1% BSA was mixed with different concentrations of fucoidan. The mixture was incubated in the wells coated with type II TGF-β receptor, and the amount of r-TGF-β1 bound to its receptor was measured by ELISA (*$p < 0.05$ compared with L-F; **$p < 0.05$ compared with L-F and M-F; $n = 6$). (B) Recombinant LTGF-β1 was mixed with different concentrations of fucoidan before heat-activation, and activated TGF-β1 was measured by ELISA. In another sample[@], recombinant LTGF-β1 was subjected to heat-activation and thereafter mixed with fucoidan to estimate the decrease in TGF-β1 through interaction with activated TGF-β1 ($n = 9$). (C) HK-2 cell culture supernatant was mixed with different concentrations of fucoidan before acid-activation, and activated TGF-β1 was measured by ELISA. In another sample[@], the culture supernatant was subjected to acid-activation and thereafter mixed with fucoidan to estimate the decrease in TGF-β1 through interaction with activated TGF-β1 ($n = 7$). The differences (a–d) of the measured TGF-β1s when fucoidan was added before and after activation procedure were regarded as the portion of inhibited activation by fucoidan. (*$p < 0.05$ compared with a; **$p < 0.05$ compared with c.)

Recombinant small LTGF-β1 was acid-activated after preincubation with different concentrations of FF7 for 10 min and the level of active TGF-β1 was measured. As shown in Fig. 2A, FF7 inhibited acid-activation of recombinant small LTGF-β1. To investigate the interaction at neutral pH as in physiologic condition, we also evaluated the effect of FF7 on heat-activation of recombinant small LTGF-β1 at neutral pH. As shown in Fig. 2B, heat-activation was also inhibited by FF7.

Large LTGF-β1 released in the culture supernatant of HK-2 cells was readily activated by acid treatment, but not by heat treatment. As in the activation of recombinant LTGF-β1, acid activation of large LTGF-β1 of HK-2 cells was also inhibited by FF7 (Fig. 2C).

3.3. The strengths of interactions of fucoidan with TGF-β1 and LTGF-β1 are dependent on its MW

Crude fucoidan was divided into three fractions; high, middle and low MW. High MW fucoidan inhibited the receptor binding of TGF-β1 more efficiently than middle or low MW fucoidan (Fig. 3A). The effect of fucoidan on activation of LTGF-β1 was also dependent on its molecular size. As molecular weight increases, fucoidan had more inhibitory effect on the activation of small recombinant LTGF-β1 or large LTGF-β1 of HK-2 cells (Fig. 3B and C).

3.4. Fucoidan inhibits enzymatic activation of platelet LTGF-β1

After isolation of platelets from whole blood, we further evaluated whether fucoidan inhibits the activation of LTGF-β1 that are released by hypotonic lysis of platelets. For this, platelet lysates were mixed with middle MW fucoidan at doses of 0, 10, 25 and 50 μg/ml and incubated at room temperature for 1 h. As shown in Fig. 4A, the level of active TGF-β1 was decreased in a dose-dependent manner by fucoidan. To assess the reduced portion of the measured TGF-β1 by interaction of fucoidan with activated TGF-β1, the same platelet lysates (1/10 dilution) were acid-activated, and then mixed with middle MW fucoidan at doses of 0, 10, 25 and 50 μg/ml, and TGF-β1 was measured. Fucoidan had no effect on it (Fig. 4B).

As shown in Fig. 4C, activation of platelet LTGF-β1 was significantly inhibited by furin inhibitors or the furin substrate, suggesting furin-like proprotein convertase is involved in this process. To evaluate whether the inhibitory effect of fucoidan is related with the interaction with furin-like proprotein convertase, the enzyme activity was determined by measuring the release of the fluorescent AMC moiety. As shown in Fig. 4D, platelet lysates contained furin-like proprotein convertase activity which was inhibited by both furin inhibitors. In contrast, the fluorescent intensity increased as the dose of fucoidan increases.

4. Discussion

In the present study, we explored the biologic effects of interactions of fucoidan with TGF-β1 and LTGF-β1. Our data demonstrate that fucoidan binds to small and large LTGF-β1 as well as TGF-β1, and inhibits activation of LTGF-β1 and the receptor binding of TGF-β1.

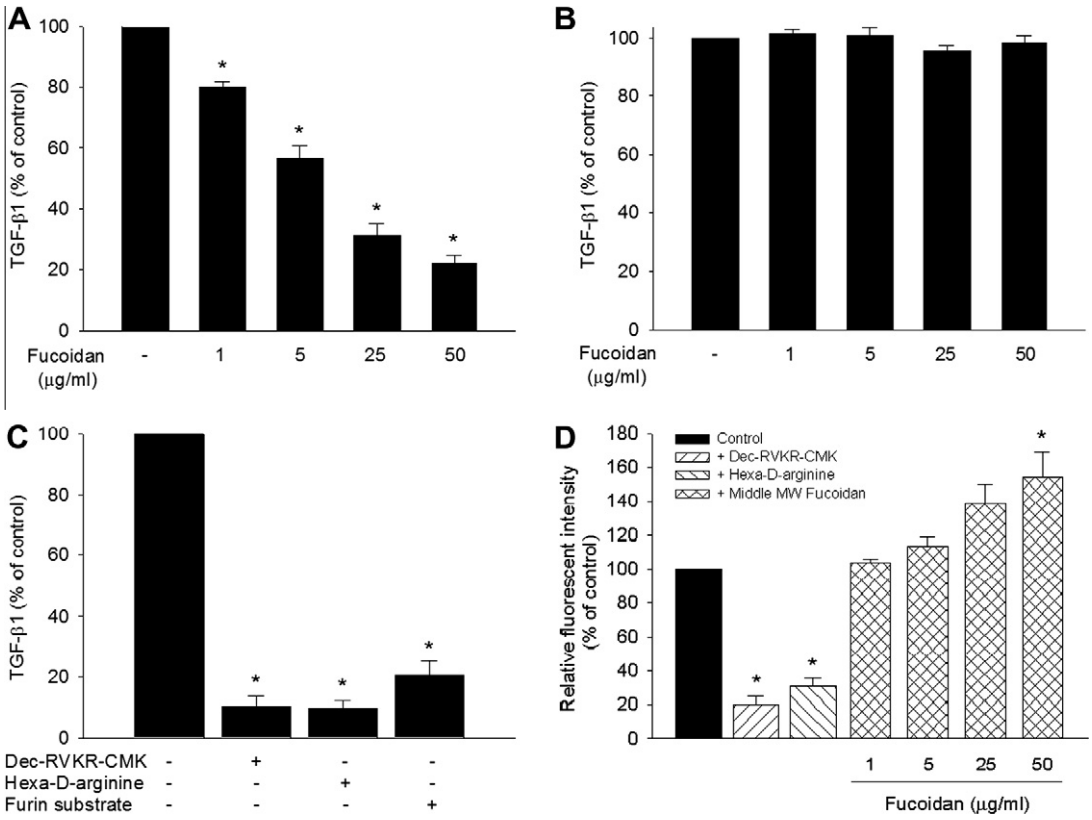

Fig. 4. Fucoidan inhibits enzymatic activation of platelet LTGF-β1. (A) Platelet lysates prepared by hypotonic lysis were mixed with middle MW fucoidan at doses of 0, 1, 5, 25 or 50 μg/ml, and incubated at room temperature for 1 h to allow enzymatic activation of LTGF-β1. TGF-β1 was measured by ELISA (*$p < 0.05$ compared with control; $n = 6$). (B) Platelet lysate was treated with acid to activate LTGF-β1 and thereafter mixed with different concentrations of middle MW fucoidan. TGF-β1 was measured by ELISA ($n = 6$). (C) Platelet lysates were treated with furin inhibitors (Dec-RVKR-CMK and Hexa-D-arginine) or furin substrate and incubated at room temperature for 1 h to allow LTGF-β1 activation. TGF-β1 was measured by ELISA (*$p < 0.05$ compared with control; $n = 6$). (D) Furin-like proprotein convertase activity in platelet lysate was assayed using a fluorogenic furin substrate in the presence or absence of Dec-RVKR-CMK (100 μM), hexa-D-arginine (100 μM), or middle MW fucoidan. (*$p < 0.05$ compared with control; $n = 7$).

Fucoidan is known to bind to TGF-β1 [16], but it has not been clearly elucidated whether TGF-β1 bound to fucoidan has normal biological activity. In a previous study [16], fucoidan was shown to protect TGF-β1 from proteolytic degradation, and potentiated TGF-β1 activity as measured by its ability to inhibit proliferation of mink lung epithelial cells (CCL64). In contrast, our data revealed that TGF-β1 bound to fucoidan is unable to interact with its receptor, and thus has no biologic activity, as evidenced by the following findings; measurement of TGF-β1 by ELISA using the wells coated with type II TGF-β receptor was inhibited by fucoidan in a dose-dependent manner. In another experiment, fucoidan inhibited binding of ^{121}I-TGF-β1 to type II TGF-β receptor coated on the well plate. Pretreatment of type II TGF-β receptor with fucoidan did not alter the binding of ^{121}I-TGF-β1 to TGF-β receptor, suggesting that the inhibitory effect of fucoidan occurs by interaction with TGF-β1, but not with the TGF-β receptor. Moreover, fucoidan attenuated the cellular effect of TGF-β1 as measured by phosphorylation of Smad2 in HK-2 cells. This is in agreement with another study that fucoidan abrogated anti-proliferative effect of TGF-β1 in cultured dermal fibroblast [17]. The reasons for the contradictory findings are not clear, but there is a possibility that fucoidan might inhibit CCL64 proliferation independently of TGF-β1, which could be falsely counted as TGF-β1 activity, considering that it has an anti-proliferative effect on some types of cells including smooth muscle cells [20].

So far, there has been no data regarding the interaction between fucoidan and LTGF-β1. In this study, fucoidan interacted with platelet LTGF-β1 and inhibited enzymatic activation of it. This was not by inhibition of furin-like proprotein convertase, the enzyme responsible for activation of platelet LTGF-β1. Rather, fucoidan caused an increase in the release of the fluorescent AMC moiety from the furin substrate. As LTGF-β1 and the furin substrate compete for furin-like proprotein convertase, it is likely that blocking of LTGF-β1 by fucoidan leads to increased availability of the enzyme to the furin substrate. Acid- or heat-activations of small recombinant LTGF-β1 and large LTGF-β1 of HK-2 cells were also inhibited by fucoidan. Though the interaction of fucoidan and LTGF-β1 was not evaluated in LTGF-β1 activation by other proteolytic enzymes or by conformational changes of the LAP, these findings suggest that the interaction renders LTGF-β1 resistant to activation.

Crude fucoidan is a mixture of varying sized molecules, with a wide range of MW [18]. The strength of interaction between fucoidan and LTGF-β1 or TGF-β1 was dependent on the MW of it. As MW of fucoidan increases, it had more potent interactions with LTGF-β1 and TGF-β1, resulting in greater inhibitions of LTGF-β1 activation and TGF-β1 receptor binding.

Besides TGF-β1, fucoidan is known to bind to P-selectin [21], fibronectin [22] and platelet factor 4 [23], which are present in α-granules of platelets. In this study, middle MW fucoidan (50 μg/ml) inhibited the receptor binding of r-TGF-β1 in PBS/0.1% BSA by ~25%, but failed to decrease active TGF-β1 in acid-activated platelet lysate. This may occur because competitive binding to other proteins in the platelet lysate makes fucoidan less available to TGF-β1. In contrast, middle MW fucoidan significantly inhibited LTGF-β1 activation in platelet lysate. This suggests that LTGF-β1 has a higher affinity for middle MW fucoidan than does TGF-β1.

Recent studies have shown that fucoidan has antifibrotic effects. In Sprague–Dawley rats, fucoidan was shown to attenuate CCl_4-induced hepatic fibrosis [14]. It also reduced the infarct scar size in a rat model of myocardial ischemia–reperfusion injury [15]. TGF-β1 has a prominent role in the formation of peritoneal adhesion bands [24]. In a rat model, local administration of fucoidan film greatly reduced postsurgical adhesion [25]. The inhibitory effects of fucoidan on LTGF-β1 and TGF-β1 shown in this study may in part account for its antifibrotic effects.

In conclusion, fucoidan binds to LTGF-β1 and TGF-β1, and inhibits its LTGF-β1 activation and TGF-β1 receptor binding. The inhibitory effects were greater with higher MW of fucoidan.

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