Fucoidan Is the Active Component of Fucus vesiculosus that Promotes Contraction of Fibroblast-Populated Collagen Gels

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The fibroblast-populated collagen gel culture method has been evaluated as a dermal model of wound contraction and granulation in tissues during the wound healing process and as an in vitro model of dermal tissue. We previously reported that an extract of Fucus vesiculosus promoted fibroblast-populated collagen gel contraction and that the promotion of the gel contraction was due to the increased expression of integrin α2β1 on the surface of the fibroblasts. In this study, we investigated the active component of the extract of this alga using extraction and fractionation techniques. Water extraction of the alga was followed by precipitation with excess ethanol and then gel filtration with the boundary molecular weight of 30000. The high molecular weight fraction obtained from gel filtration was fractionated by ion exchange chromatography on diethylaminoethyl cellulose column to give active fractions that have more polar properties. These polar, high molecular weight fractions which contained molecules with fucose and sulfate groups showed significant gel contraction-promoting activity and integrin expression-enhancing activity, and were estimated to be the sulfated-polysaccharide fucoidan. Commercially available fucoidan showed similar activities to the above-described fraction of this alga. Although it remains necessary to precisely identify the specific active component, the results indicate that fucoidan is the active component which promotes collagen gel contraction, and also indicate the possibility that it dose so by enhancing the integrin α2β1 expression.

Key words collagen gel; fibroblast; integrin; contraction; fucoidan

The fibroblast-populated collagen gel culture method has been evaluated as a dermal model of wound contraction and granulation in tissues during the wound healing process and as an in vitro model of dermal tissue. It is well known that the collagen gel contraction is promoted not only by FCS (fetal calf serum) but also by various peptide and protein molecules such as TGFβ (transforming growth factor β), endostatin, PDGF (platelet-derived growth factor) and thrombin. However, all of them are in vivo cytokines or growth factors closely involved in wound contraction and granulation. Other than peptides and protein molecules, only a few compounds such as retinooids are known to promote the collagen gel contraction. We recently reported the effects of various natural product extracts on collagen gel and mechanical properties using this model, and observed that an extract of Fucus vesiculosus promoted gel contraction and increased the relaxation time of the gels. In addition, we investigated the mechanism of the promotion of the gel contraction, noting increased expression of integrin α2β1 molecules on the surface of the fibroblasts, which suggested that the extract of Fucus vesiculosus promoted the contraction by increasing the expression of integrin molecules on the fibroblast's surface.

In this study, we investigated the active component of the extract of Fucus vesiculosus which promotes collagen gel contraction, using extraction and fractionation techniques, and determined fucoidan to be the active component of this alga.

MATERIAL AND METHODS

Antibodies, Collagen and Chemicals Mouse anti-human integrin α2β1 (monoclonal antibody) was purchased from Gibco BRL, and FITC (fluorescein isothiocyanate)-labeled anti-mouse IgG was from Nordic Immunology. Collagen solution (Cell matrix Type I-A) was purchased from Nitta Gelatin Co. Ltd., and collagenase was from Wako Pure Chemical Industries, Ltd. Fucoidan from the brown seaweed Fucus vesiculosus and β-(++)-fucose were obtained from Sigma Chemical Co. Fucosterol and sodium alginate (KF-0000-81) were from Funakoshi Co., Ltd.

Extraction of Fucus vesiculosus Extracts of dried Fucus vesiculosus obtained using various solvents were purchased from Ichimaru Pharmacos Co., Ltd. (specially ordered). The extract solvents and residue weight % of extracts are summarized in Table 1. The extracts were used at concentrations from 0.01 to 0.0001% (residue weight %), except for the 100% ethanol extract, 100% 1,3-butyleneglycol (BG) extract and 100% 1,3-propyleneglycol (PG) extract, which were at less than 0.001% concentration because of low residue content.

Fractionation of Fucus vesiculosus Extract Procedures

Table 1. Residue Weight % of the Extracts of Dried Fucus vesiculosus Obtained Using Various Solvents

<table>
<thead>
<tr>
<th>Extraction solvent</th>
<th>Residue weight %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water extract</td>
<td>1.61</td>
</tr>
<tr>
<td>Hot water extract</td>
<td>2.21</td>
</tr>
<tr>
<td>50% ethanol extract</td>
<td>1.1</td>
</tr>
<tr>
<td>50% BG extract</td>
<td>1.0</td>
</tr>
<tr>
<td>50% PG extract</td>
<td>1.1</td>
</tr>
<tr>
<td>100% ethanol extract</td>
<td>0.041 b</td>
</tr>
<tr>
<td>100% BG extract</td>
<td>0.036 b</td>
</tr>
<tr>
<td>100% PG extract</td>
<td>0.050 b</td>
</tr>
</tbody>
</table>

a) BG and PG represent 1,3-butyleneglycol and 1,3-propyleneglycol, respectively.
b) These extracts were used at below 0.001% concentration (residue weight %).

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Dried *Fucus vesiculosus* Extract

Extracted with water for 24 h at room temperature with stirring. Filtration.

Crude extract (100 %)  →  Residue

Precipitated by adding 900 volume % of ethanol. Stirred and kept at room temperature for 12 hours. Filtration.

Precipitate  →  Supernatant F1 (42.5 %)

Suspended in 90 volume/volume % ethanol with stirring. Centrifuged (2000 rpm, 15 minutes)

Precipitate  →  F2 (50.1 %)  →  Supernatant (waste)

Solvent removed at reduced pressure, and dissolved in water. Gel filtration on Centriprep YM-30 (Mw 30000)

High molecular weight fraction (Mw > 30000)  →  F4 (10.0 %)  →  (Mw < 30000)  →  F3 (40.1 %)

Eluted in DEAE (Toyopearl 650M) column stepwise with 0.25 M to 2.0 M NaCl to give the six respective fractions

F5  →  F6  →  F7  →  F8  →  F9  →  F10

Monitored by the phenol-H$_2$SO$_4$ method.

**Chart 1. Fractionation Procedure of *Fucus vesiculosus* Extract**

High molecular weight fraction (Mw > 10000)  →  F12

Low molecular weight fraction (Mw < 10000)  →  F11

**Chart 2. Gel Filtration of Fraction F2 with Boundary Molecular Weight 10000**

used to fractionate the *Fucus vesiculosus* extracts are summarized in Chart 1. Dried *Fucus vesiculosus* was extracted with water for 24 h with stirring at room temperature, the residue was removed by filtration, and then 900 v/v% of ethanol was added to the filtrate, and the mixture was stirred and kept for 12 h at room temperature. The precipitate was collected by filtration, then suspended in 90 v/v% of ethanol, stirred, and centrifuged (2000 rpm) for 15 min to give the precipitate fraction F2. The filtrate was concentrated at reduced pressure and dried to give the supernatant fraction F1.

The ethanol contained in F2 was removed at reduced pressure and the precipitate was dissolved in water and then gel-filtered on a Centriprep YM-30 (30000 nominal molecular weight (MW) limit, Millipore Corp.) using water as the solvent. High molecular weight fraction F4 (MW>30000) and low molecular fraction F3 (MW<30000) were obtained. The yields of fractions F1, F2, F3, and F4 from the crude water extract were 42.5, 50.1, 10.0 and 40.1%, respectively.

Fractions F11 and F12 were obtained by gel-filtration of fraction F2 on Centriprep YM-10 (10000 nominal MW limit, Millipore Corp.) using water as the solvent (Chart 2).

Fraction F4 (1.0 g) was fractionated by ion exchange chromatography on a diethylaminoethyl cellulose (DEAE) column (Toyopearl 650M) by stepwise elution with 0.25, 0.5, 0.75, 1.0, and 2.0 M NaCl to give the six respective fractions F5 (approximately 300 mg), F6 (approximately 200 mg), F7 (approximately 167 mg), F8 (120 mg), and F10 (24 mg) as illustrated in Chart 1 and Fig. 1. The carbohydrates in the column elutes were monitored by the phenol-H$_2$SO$_4$ method.

**Fig. 1. DEAE Column Chromatography of Fraction F4**

Fraction F4 was fractionated by chromatography on a DEAE column to give six respective fractions. The carbohydrates in column elutes were monitored by the phenol-H$_2$SO$_4$ method.

**Cell Culture** Human fibroblasts were purchased from Dainihon Pharmaceutical Co., Ltd., and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FCS as previously described. Fibroblasts from the third to the eighth passage were used in this study.

**Preparation of Fibroblast-Populated Collagen Gel** Fibroblast-populated collagen gels were prepared as previously described. Briefly, 5 volumes of collagen solution, 2 volumes of 5× concentrated DMEM and 1 volume of 250 mM HEPES-2.2% NaCO$_3$ solution, 0.5 volume of FCS, the required amount of distilled water and a 1—2 ml aliquot of fibroblast suspension in 5% FCS-DMEM (2×10$^6$ cells/ml) were gently mixed at 4°C giving a cell suspension at a final density of 5×10$^6$ cells/ml and a 1.5 mg/ml collagen concentration. Each extract was added to the collagen solution and incubated in culture dishes at 37°C. After 18—24 h, 0.5 ml of the medium was added to each of the formed collagen gels, which were then scraped off at the periphery to release the gels from the wells (day 0).

**Measurement of Gel Contraction (Gel Volume)** Gel contraction was determined by measuring the gel volume as previously described. Briefly, gels that had been incubated with fibroblasts for 5 d were fixed in 8% buffered formalin (Mildform 20 VM, Wako Pure Chemical Industries, Ltd.) at 4°C for 24 h and the gel weight was taken as indicating the gel volume.

**Determination of Cell Number** Fibroblast-populated collagen gels were treated with *Fucus* extracts, separated fractions or fucoidan for 5 d. Each gel consisted of 3 ml of the collagen solution containing 5×10$^6$ cells/ml; hence, one gel included 1.5×10$^6$ cells before incubation. The cell number in the collagen gel was determined as previously described. Briefly, the gels were digested with collagenase, and viable fibroblasts were counted after staining with trypan blue in a hemocytometer.

**Flow Cytometry Analysis of Surface Integrins of Fibroblasts** Monolayers of cultured fibroblasts were treated with *Fucus* extracts, separated fractions or fucoidan at vari-
ous concentrations at 37°C for 48 h, and the cells were harvested by trypsin/EDTA treatment. The cells were washed with phosphate buffered saline (PBS) (containing 0.1% FCS) and 0.02% sodium azide, and incubated with control mouse-IgG (10 μg/ml) as a blank control experiment, or mouse antihuman integrin α2β1 (1:100) for 60 min on ice. After the cells were washed twice with PBS, they were labeled with FITC-labeled anti-mouse IgG (1:100) for 60 min. They were again washed twice with PBS, and fluorescence staining was analyzed using a flow cytometry system (Becton Dickinson). Analysis was based on data collected from >10000 events, and values were expressed as the MFI (mean of fluorescence intensity) of the gated cell population. Dead cells were identified by propidium iodide uptake.

Statistical Analysis  Student’s t-test was used for statistical analysis, and p<0.05 was considered significant. All experiments were performed at least twice unless otherwise stated, and data of one representative experiment are shown in tables and figures.

RESULTS

Effect of Extraction Solvent  Figure 2 shows the effects of various extraction solvents on the collagen gel contraction expressed as relative values. Among the extracts prepared with the various solvents tested, in particular the water extract and hot water extract significantly promoted the collagen gel contraction, as indicated by a decrease of gel volume. Moreover, the extracts made with the solvents containing water, such as 50% ethanol or 50% BG extracts, showed moderate contraction-promoting activity.

Ethanol Precipitation and Gel Filtration  As shown in Fig. 3, fraction F2 obtained by precipitation with ethanol promoted the collagen gel contraction as well as the crude water extract. In contrast, the ethanol-soluble filtrate fraction F1 had no promoting activity. The high molecular weight fraction F4 obtained by gel filtration of fraction F2 significantly promoted the collagen gel contraction, but low molecular weight fraction F3 had little activity.

Fractionation by DEAE Column and Determination of Fucoidan  Fraction F4 was eluted from the DEAE column with NaCl solutions to give six respective fractions (Chart 1 and Fig. 1). As shown in Fig. 4, fractions F5, F6, and F7, which had relatively low polar properties, had little effect on collagen gel contraction. On the other hand, fractions F8, F9, and F10, which have more polar properties, significantly promoted the contraction. Hydrolysis of the fraction F8 in 90% formic acid at 100°C for 24 h produced a certain amount of fucose, and infrared analysis (KBr pellet) of this fraction showed the presence of hydroxyl and sulfate groups. Major absorption bands of fraction F8 were observed at about 3500 cm⁻¹ (broadband, O-H stretching), 1030–1165 cm⁻¹ (broadband, hemiacetal stretch-
Fig. 5. Effects of Fucoidan (Sigma) and Water-Extracted Fucus on the Contraction of Fibroblast-Populated Collagen Gels

Fibroblast-populated collagen gels were treated with each fraction at concentrations from 0.01% to 0.0001% (residue weight %). All measurements were carried out using gels that had been incubated with fibroblasts for 5d. Each value represents the mean ± S.D. Significantly different from control. ** p<0.01.

Fig. 6. Effect of Lower or Higher Molecular Weight Fractions, F3, F4, F11, and F12, of Fucus vesiculosus on the Integrin α2β1 Expression on the Cell Surface of Monolayer Cultured Fibroblasts

Fractions F3 and F4 were separated by gel filtration with the boundary molecular weight of 30000, and fractions F11 and F12 were separated with the boundary molecular weight of 100000.

ing), and 1250—1270 cm⁻¹ (broadband, S=O stretching). Absorption at 1645 cm⁻¹, 840 cm⁻¹, 820 cm⁻¹ was also observed. These infrared absorption properties of fraction F8 were similar to those published for fucoidan.9,9 After Fraction F9 had a similar infrared absorption spectrum.

Effect of Fucoidan on Collagen Gel Contraction As shown in Fig. 5, fucoidan significantly promoted the collagen gel contraction, as did the water extract of Fucus vesiculosus even at concentrations as low as 0.0001%.

Effect of Separated Fractions of Fucus vesiculosus and Fucoidan on the Expression of Surface Integrin α2β1 in Fibroblasts As shown in Fig. 6, fucoidan and the water extract of Fucus vesiculosus significantly increased the expression of surface integrin α2β1 on the fibroblasts. Fraction F3 (MW<30000) and fraction F4 (MW>30000) obtained by gel filtration increased the integrin α2β1 expression in a similar manner at concentrations from 0.01 to 0.03% (residue weight %). On the other hand, fraction F11 (MW<10000) showed no effect on the expression of integrin α2β1, whereas F12 (MW>10000) significantly increased the expression of surface integrin α2β1 on the fibroblasts.

Effect of Water Extract of Fucus vesiculosus and Fucoidan on Proliferation of Fibroblasts Neither the water extract of Fucus nor fucoidan affected the cell proliferation in collagen gels (data not shown in Figure). Each gel consisted of 3 ml of the collagen solution containing 5×10⁶ cells/ml; hence, one gel included 1.5×10⁶ cells before incubation. After 5d of culture, the cell numbers in the gel without treatment (control), treated with water extract (at concentration of 0.001%), or with fucoidan (at 0.001%) were 1.25±0.20, 1.28±0.15, and 1.31±0.27×10⁶ cells/gel (mean±S.D.), respectively. These results showed that there were no significant effects on the cell proliferation.

DISCUSSION

The brown seaweed Fucus vesiculosus is a common littoral alga of the coasts of the Northern Atlantic, the Pacific Ocean, and the Baltic Sea. It is well known that water extracts from this alga show various biological activities such as a potent anticoagulant activity.10—13 We previously reported that the alga promotes fibroblast-populated collagen gel contraction, and that this promotion is due to the increased expression of integrin α2β1 on the fibroblast surface.7 In this report, we clarified that the active component of this alga is fucoidan, which is a unique polysaccharide composed mainly of fucose and sulfate.

The evaluation of the effect of the solvent used to extract this alga on the gel contraction-promoting activity showed that the water extract and hot water extract had higher activity than extracts made with other solvents (Fig. 2). As shown in Fig. 3, fraction F2 precipitated by the addition of ethanol to this water extract showed higher activity than the supernatant fraction F1. Moreover, the higher molecular weight fraction F4 obtained by gel filtration of fraction F2 showed stronger activity than the low molecular weight fraction F3. These results indicate that the active component of Fucus vesiculosus on the gel contraction-promoting activity has a relatively high molecular weight and is relatively hydrophilic.

Fractions F8—F10 obtained by further fractionation of F4 by DEAE column chromatography showed stronger gel contraction-promoting activity than fractions F5—F7 (Fig. 4). Fractions F5, F6, and F7, which have relatively less polar properties, were analyzed to estimate their contents of various carbohydrates, polysaccharides or alginates. The polar fraction F8, which contained sulfate groups and fucose, as described in the results above, was identified as sulfated-polysaccharides. Fractions F9 and F10 were also identified as sulfated-polysaccharides, very similar to fraction F8. Chemical composition studies have indicated that the sulfated-polysaccharide fucoidan, various phlorotannins, polyphenols, alginates, and fucosterol are the most abundant constituents of this alga.11—13 Collectively, these results strongly suggest that the more polar fractions F8, F9, and F10 contain fucoidans, which comprise a wide spectrum of fucans containing mainly fucose and sulfate.14 The differences of polarity among fractions F8, F9, and F10 are believed due to the proportion of sulfate residues contained in a molecule of fucoidan.9

Because fucoidan from Fucus vesiculosus is commercially available, we investigated the effect of this fucoidan on the collagen gel contraction promoting activity. As shown in Fig. 5, fucoidan showed significant gel contraction-promoting activity at concentrations from 0.001% to 0.0001% (residue weight %), which indicates that it has appreciably higher activity than the crude water extract of this alga. α-(+)-Fucose
(at concentrations from 0.01 to 1 mM), sodium alginate (at concentrations from 0.01 to 1 mM) and fucosterol (at concentrations from 1 to 100 μM) had no activity (data not shown).

Next, we investigated the mechanism of the gel contraction-promoting effects of the extract of this alga and fucoidan focusing on integrins. It is well known that the integrin molecules, in particular the integrin α2β1 heterodimer that mediates collagen-fibroblast interaction, play an important role in collagen gel contraction activity.\textsuperscript{15,16} We previously reported that an extract of this alga enhances the expression of fibroblast integrins, resulting in promotion of gel contraction.\textsuperscript{17}

As shown in Fig. 6, the water extract of Fucus and fucoidan (commercially available) significantly enhanced the integrin expression. Both fractions F3 and F4, which were separated by gel filtration with the boundary molecular weight of 30000, also significantly enhanced the expression. However, fraction F3 did not show any gel contraction-promoting activity, which is not consistent with the results of the integrin expression.

Nishino et al. made a precise analysis of the components of commercially available fucoidan and reported that fucoidan has a wide range of molecular weight, from 6.8—680 thousand.\textsuperscript{18} Therefore, we investigated the activities of fractions F11 and F12, which were separated by gel filtration with the boundary molecular weight of 10000. Fraction F12, which consists of molecules with molecular weight greater than 10000, showed strong integrin expression-enhancing activity, but fraction F11 with molecules with molecular weight less than 10000 did not show this activity. These results suggest that the lower molecular weight fucoidan (theoretically, 10000<MW<30000) contained in fraction F3 should have an integrin expression-enhancing activity. The lack of gel contraction-promoting activity of fraction F3 is believed to be caused by the effects of other lower molecular weight components which do not enhance the integrin expressions.

Fraction F8, which was thought to contain fucoidan, was not examined, but the above results suggest that there is a relationship between the enhancement of integrin α2β1 expression and the collagen gel contraction-promoting activity of Fucus vesiculosus. Collectively, we believe that the fucoidan is the active component that promotes collagen gel contraction, and also indicate the possibility that fucoidan promotes gel contraction by enhancing the integrin α2β1 expression.

Fucoidan has been studied for its biological activities of anti-coagulant activity,\textsuperscript{19} anti-thrombin activity,\textsuperscript{20} human T-cell mitogen activity,\textsuperscript{21} anti-human immunodeficiency virus activity,\textsuperscript{20,21} and sperm-egg binding inhibition activity.\textsuperscript{21} Therefore, many investigators are interested in the fraction as a biologically active sulfated polysaccharide. The specific active component was not identified in these biological activities, however, because of the complex chemical structure: wide range of molecular weight, type of bonding, and proportion of sulfate residues. In our study, neither the specific active component related to the gel contraction nor the integrin expression was identified. Further investigation is necessary to identify this component based on the chemical structure of fucoidan.\textsuperscript{8}

REFERENCES