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The potent activity of sulfated polysaccharide, ascophyllan, isolated from *Ascophyllum nodosum* to induce nitric oxide and cytokine production from mouse macrophage RAW264.7 cells: Comparison between ascophyllan and fucoidan

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Abstract

Ascophyllan isolated from the brown alga *Ascophyllum nodosum* is a fucose-containing sulfated polysaccharide, which has similar but distinct characteristic monosaccharide composition and entire chemical structure to fucoidan. In this study, we examined the effects of ascophyllan, fucoidan isolated from *A. nodosum* (A-fucoidan), and fucoidan from Sigma (S-fucoidan) as a representative fucoidan derived from other source (*Fucus vesiculosus*) on mouse macrophage cell line RAW264.7 cells. No significant cytotoxic effects of ascophyllan and A-fucoidan on RAW264.7 cells were observed up to 1,000 $\mu\text{g/ml}$, while S-fucoidan showed cytotoxic effect in a concentration-dependent manner. Ascophyllan induced extremely higher level of nitric oxide (NO) production from RAW264.7 cells than those induced by fucoidans over the concentration range tested (0-200 $\mu\text{g/ml}$). Reverse transcription polymerase chain reaction (RT-PCR) and western blot analysis revealed that expression level of inducible NO synthase (iNOS) in ascophyllan-treated RAW264.7 cells was much higher than the levels detected in the cells treated with fucoidans. Furthermore, the activities of ascophyllan to induce the secretion of tumor necrosis factor- α (TNF- α) and granulocyte colony-stimulating factor (G-CSF) from RAW264.7 cells were also greater than those induced by fucoidans especially at lower concentration range (3.1~50 $\mu\text{g/ml}$). The activities of ascophyllan to induce NO and cytokine production in mouse peritoneal macrophages were also stronger than those of fucoidans. Electrophoretic mobility shift assay (EMSA) using infrared dye labeled nuclear factor-kappa B (NF- κB) and AP-1 consensus sequences suggested that ascophyllan can strongly activate these transcription factors. Marked increase in the nuclear translocation of p65, and the phosphorylation and degradation of I κB - α were also observed in ascophyllan-treated RAW264.7 cells. Analysis using mitogen-activated protein (MAP) kinase inhibitors and western blot analysis suggested that c-Jun N-terminal kinase (JNK) and p38 MAP kinase are mainly involved in ascophyllan-induced NO production.

Key words: *Ascophyllum nodosum*; ascophyllan; RAW264.7 cells; nitric oxide; TNF- α , G-CSF; NF- κ B; AP-1; MAP kinase signaling pathway

Introduction

Many marine resources, especially marine algal polysaccharides such as alginate, fucoidan, carrageenan, laminaran, and agar have recently been drawn a great attention from diverse research fields to develop as new drugs and health foods or supplements. Some of these polysaccharides have been reported to have antitumor [1, 2, 3], antiviral [4, 5], anticomplementary [6, 7], anticoagulant [8, 9], antioxidant [10, 11], anti-inflammatory [12], and immuno-modulatory [13] activities. Even a description of the use of seaweed products for medical purposes is found in traditional Chinese herbal medicine as early as the sixteenth century [14, 15].

Brown seaweeds such as *Fucus vesiculosus*, *Ascophyllum nodosum*, and *Laminaria angustata* are often used as raw materials for the preparation of alginate, laminaran, and fucoidan. Fucoidan is basically a sulfated fucan containing fucose as a main component and some times containing uronic acids, galactose, and xylose as minor compositions [16]. Since fucoidans have structural similarity to heparin, they are well-known to have potent anticoagulant [8] and antithrombotic activities [8, 17], and recent studies have demonstrated that fucoidans have numerous other biological properties such as antiviral [18, 19], antitumor, anti-inflammatory [20, 21], immuno-modulatory [22], and apoptosis-inducing activities [23, 24].

In addition to alginate and fucoidan, it has been reported that *A. nodosum* contains ascophyllan (xylofucoglycuronan) as a sulfated fucan preparation distinguished from fucoidan [25, 26]. In our previous study, the presence of ascophyllan was confirmed as a separated fraction from the fucoidan fraction in the extracts prepared from *A. nodosum* [27]. Regarding the biological activities of ascophyllan, we found that ascophyllan has a growth-promoting activity on MDCK cells, while fucoidan was rather toxic to this cell line. This result clearly indicates that there is a difference in the bioactivities of ascophyllan and fucoidan at least in the effects on the growth of MDCK cells [27]. Furthermore, we found that both ascophyllan and fucoidan isolated from *A. nodosum*

showed similar concentration-dependent cytotoxic effects on U937 cells, and ascophyllan was capable of inducing the secretion of tumor necrosis factor- α (TNF- α) and granulocyte colony-stimulating factor (G-CSF) from mouse macrophage cell line RAW264.7 [28]. These results suggest that ascophyllan is an attractive bioactive polysaccharide with multiple bioactivities for the applications as supplement or pharmaceutical agents.

Macrophages play important roles in host defense system, and they produce various inflammatory mediators and cytokines. Nitric oxide (NO), known to be an inflammatory mediator, is produced by the activation of inducible nitric oxide synthase (iNOS) from L-arginine in macrophages [29, 30]. NO is also generated by many cell types in a variety of tissues, and this gaseous mediator acts as a vascular relaxing agent, a neurotransmitter, inflammatory mediator, and specific immunity regulator [30]. It is well documented that a stimulatory pathway in macrophages is initiated by binding of a bacterial cell wall component (lipopolysaccharide, LPS) to the CD14-toll-like receptor that triggers a complex kinase cascade and eventually leads to gene activation and subsequent expression of iNOS [31]. NO has been known to be an essential cytotoxic factor in the killing of pathogens and tumoricidal agent as well [30, 32]. It has recently been reported that fucoidan induces NO production in RAW264.7 cells via p38 mitogen-activated protein kinase (MAP kinase) and NF- κ B-dependent signaling pathways through macrophage scavenger receptors [33], while another study found that blocking of macrophage scavenger receptors did not result in the inhibition of the fucoidan activity, suggesting that such receptor is not involved in fucoidan action [34]. Recent studies have reported that fucoidan rather inhibits the release of NO from LPS-stimulated RAW264.7 cells [35, 36]. Thus, the precise action mechanism of fucoidan in terms of NO production in macrophages remains a matter of debate.

In this study, we investigated the effects of ascophyllan on RAW264.7 cells in terms of NO and cytokine induction, and compared the activities with those of fucoidans prepared from *A. nodosum* and *F. vesiculosus*.

Materials and methods

Materials

Fucoidan, purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA), was named as S-fucoidan to distinguish from the fucoidan (A-fucoidan) isolated from *A. nodosum*. A Cell Counting Kit-8 (WST-8) was obtained from Dojindo Chemical Laboratories (Kumamoto, Japan). PD98059, SB202190 and SP600125, which are specific inhibitors for extracellular-regulated kinase (ERK), p38 mitogen-activated protein (MAP) kinase, and c-jun N-terminal kinase (JNK), respectively, were purchased from Wako Pure Chemical industries, Ltd (Osaka, Japan). Other chemicals were of the highest grade commercially available.

Preparation of ascophyllan and fucoidan from A. nodosum

A. nodosum collected at the coast in Norway was obtained from KAISEI Co. Shimonoseki, Japan. Ascophyllan and fucoidan were prepared from the powdered *A. nodosum* as described previously [27, 28]. In brief, alginate fraction was removed from the hot water-extract by acid precipitation and subsequent digestion with alginate lyase. Ascophyllan and fucoidan fractions were obtained from the residual fucan fraction based on their different solubility in aqueous ethanol. Composition analysis confirmed that ascophyllan was similar but distinct from those of fucoidan fraction. To avoid the effects of endotoxin which might be contaminated in polysaccharide samples, all sample solutions were passed through an endotoxin-removal column (Detoxi-gel: Thermo Fisher Scientific Inc., Rockford, IL USA), and subsequently filtered through an endotoxin-removal filter (Zetapor Dispo: Wako Pure Chemical industries, Ltd, Osaka, Japan).

Cell culture

RAW264.7 (mouse macrophage) cells were obtained from the American Type Culture Collection (Rockville, MD, USA), and cultured at 37°C in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (100 µg/ml) in a humidified atmosphere with 5% CO₂ and 95% air. Mouse peritoneal exudate macrophages were prepared from 5 weeks old male ICR mice (Tecsam, Nagasaki, Japan) at 3 days after intraperitoneal injection of 1 KE (clinical units)/mouse of OK-432 (Chugai Pharmaceutical Co. Tokyo, Japan) followed by a lavage of the peritoneal cavity with 10 ml of Hanks' balanced salt solution (HBBS) as described previously [37]. The harvested cells were washed twice with HBBS and resuspended in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 IU/ml), and streptomycin (100 µg/ml). The cells were seeded into 96-well plates at the concentration of 1×10^5 cells/well and incubated in a humidified atmosphere with 5% CO₂ and 95% air. After overnight incubation, the medium was removed and the cells were washed vigorously 3 times with HBSS to remove non-adherent cells. The adherent cells (3.5×10^4 cells/well in 96-well plates) were used as murine primary macrophages.

Cytotoxicity assay

The cytotoxic effects of ascophyllan and fucoidans on RAW264.7 cells were measured by WST-8 assay [38]. In brief, adherent RAW264.7 cells in 96-well plates (3×10^4 cells/well) were cultured with varying concentrations of samples (0-1000 µg/ml) in the growth medium for 24 h, and then 10 µl of WST-8 assay solution was added into each well to estimate the viability of the cells. After 20 min incubation at 37°C, the absorbance was measured at 450-650 nm using a multi-well scanning spectrophotometer (Thermo Electron Co., Yokohama, Japan).

Nitrite assay for the estimation of nitric oxide (NO)

To estimate NO level in RAW264.7 cells, nitrite, a stable reaction product of NO with molecular oxygen, was measured by Griess assay as described previously [39]. In brief, adherent RAW264.7 cells in 96-well plates (3×10^4 cells/well) were treated with varying concentrations of polysaccharide samples (0-200 $\mu\text{g/ml}$) for 18 h in the growth medium at 37°C , and then the nitrite levels in the culture medium were measured. When the effects of three MAP kinase inhibitors were examined, the adherent RAW264.7 cells (3×10^4 cells per well in 96-well plates) were pre-incubated with each inhibitor at the final concentration of 10 μM for 1 h at 37°C in the growth medium, and then each polysaccharide sample (final concentration of 200 $\mu\text{g/ml}$) was added into each treated cells. After 24 hours incubation at 37°C , the nitrite levels in the culture medium of the treated cells were measured. For the time-course analysis of NO production in RAW264.7 cells treated with polysaccharide samples, adherent RAW264.7 cells in 24-well plates (5×10^5 cells/well) were treated with polysaccharide samples at the concentration of 100 $\mu\text{g/ml}$ in the growth medium at 37°C , and nitrite levels in the culture medium of the treated cells were measured every 2 h until 24 h of total incubation time.

RNA isolation, cDNA synthesis and RT-PCR for iNOS mRNA

Adherent RAW264.7 cells in 24-well plates (5×10^5 cells/well) were treated with polysaccharide samples at the concentration of 100 $\mu\text{g/ml}$ in the growth medium at 37°C . After 4 h incubation, total RNA of treated cells was isolated using Sepasol-RNA I Super (Nacalai tesque, Kyoto, Japan). Total RNA (2.5 μg) was reverse-transcribed with an oligo dT primer in a 10 μl using PrimeScript[®] 1st strand cDNA Synthesis Kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instruction. PCR was performed with 1 cycle of 70 s at 95°C , 25 cycles of 55 s at 93°C , 45 s at 61°C , 40 s at 72°C and 1 cycle of 100 s at 72°C , in a 25 μl reaction mixture containing 12.5 μl of GoTag Green Master Mix

(Promega, Madison, WI, USA), 0.5 μ l of forward and reverse iNOS primers (1 μ M each) or β -actin primers (10 pM each), 0.5 μ l of 1st strand cDNA and 11 μ l nuclease-free water. The primer sequences used were 5' -CAACCAGTATTATGGCTCCT-3' (forward) and 5' -GTGACAGCCCGGTCTTTCCA-3' (reverse) for mouse iNOS and 5' -GGAGAAGATCTGGCACCACACC-3' (forward) and 5' -CCTGCTTGCTGATCCACATCTGCTGG-3' (reverse) for mouse β -actin. The β -actin primer was used as an internal control. Each PCR reaction (10 μ l) product was run on 2% agarose gels containing 0.1 μ g/ml ethidium bromide, and the amplified products (835 bp for iNOS and 840 bp for β -actin) were observed by Light capture (ATTO Co., Tokyo, Japan).

Western blot analysis

Analysis of iNOS was performed on whole-cell extracts. Adherent RAW264.7 cells in 12-well plates (1 x 10⁶ cells/well) were treated with 100 μ g/ml of polysaccharide samples in the growth medium at 37°C. After 6 h incubation, the cells were washed three times with ice cold PBS, and were lysed with 100 μ l extraction buffer (10 mM HEPES, 150 mM NaCl, 1 mM EGTA, 1% CHAPS, and 1% Triton X-100) containing 1% of protease inhibitor cocktail (Nakalai tesque Co., Kyoto, Japan). After shaking for 30 min at 4°C, the cytosolic fraction was obtained by centrifugation at 15,000 x g for 10 min, and the protein concentrations were determined with the BCA assay kit (BIO-RAD, Hercules, CA) using BSA as the standard. The extract was mixed with the equal volume of 2 x SDS-sample buffer (4% SDS, 100 mM Tris-HCl, pH 6.8, 20% glycerol) and incubated at 95°C for 5 min. Samples containing 20 μ g of proteins were subjected to SDS-PAGE in 8% polyacrylamide gel. The proteins were then electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 1% skim milk in TBS-0.1% Tween 20 (TBST). Immunostaining of the blot was performed with anti-mouse iNOS antibody and Goat anti-rabbit IgG-horseradish peroxidase conjugate

(Upstate Biotechnology, Lake Placid, NY, USA). The blot was developed by ECL Plus western blotting detection reagents (Amersham Biosciences, Piscataway, NJ, USA). To standardize the loaded protein levels, blotting with anti- β -actin antibody was also conducted at the same time. For the analysis of MAP kinases, adherent RAW264.7 cells (4×10^6 cells/3.5 cm dish) were incubated with each polysaccharide sample at the concentration of 100 μ g/ml in the serum-free DMEM at 37°C. The whole-cell lysates were prepared from the treated cells after various incubation times. Samples containing 20 μ g of proteins were subjected to SDS-PAGE in 10% polyacrylamide gel, and western blot analysis using appropriate antibodies against nonphosphorylated- and phosphorylated-p38, -JNK, and -ERK MAP kinases were carried out by the similar way as described above. The analysis of I κ B- α and phospho-I κ B- α in cytosolic extracts and NF- κ B p65 in nuclear extracts were performed, respectively. Adherent RAW264.7 cells (4×10^6 cells/3.5 cm dish) were incubated with each polysaccharide sample at the concentration of 100 μ g/ml in the serum-free DMEM at 37°C. After 1 h incubation, the cells were washed three times with ice-cold PBS and incubated with 100 μ l ice-cold cytosol extraction buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.2% Igepal CA-630, 1 mM dithiothreitol, 20 mM β -glycerophosphate, 1mM sodium orthorandate, 0.5 mM phenylmethsulfonyl fluoride, 1 μ g/ml leupeptin, and 1 μ g/ml aprotinin) for 25 min on ice. The cytosolic extracts were collected after centrifugation at 7000 x g for 5 min at 4°C. The nuclear pellets were re-suspended in 30 μ l ice-cold nuclear extraction buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 0.45 M NaCl, 25% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethsulfonyl fluoride, 1 μ g/ml leupeptin and 1 μ g/ml aprotinin) and incubated on ice for 25 min, and the nuclear extracts were obtained after centrifugation at 15,000 x g for 10 min at 4°C. After measuring the protein concentrations, the extract was mixed with equal volume of 2 x SDS-sample buffer and incubated at 95°C for 5 min. Samples containing 20 μ g of protein were applied on 12.5% SDS-PAGE, and then electrically transferred to a polyvinylidene difluoride (PVDF) membrane. The western blot analysis of the cytosolic and nuclear extracts were

conducted by the similar way as described above except using specific anti-NF- κ B p65, anti-I κ B- α , and anti-phospho-I κ B- α antibodies which are obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA).

Enzyme-linked immunosorbent assay (ELISA)

Adherent RAW264.7 cells in 96-well plates (3×10^4 cells/well) were treated with varying concentrations of polysaccharide samples (0 to 200 μ g/ml) in the growth medium at 37°C. After 18 h incubation, the levels of TNF- α and G-CSF in the culture supernatants of treated cells were measured by sandwich ELISA with two antibodies to two different epitopes on TNF- α or G-CSF molecule as described previously [39]. The TNF- α and G-CSF concentrations were estimated from a reference to a standard curve for serial two-fold dilution of murine recombinant TNF- α or G-CSF.

Electrophoretic mobility shift assay (EMSA)

Adherent RAW264.7 cells (2×10^6 cells/3.5 cm dish) were treated with 100 μ g/ml of polysaccharide samples in serum-free DMEM at 37°C. The untreated cells were used as control. After 1 h incubation, the nuclear proteins were extracted from the cells as described previously [39, 40]. Electrophoretic mobility shift assay (EMSA) was performed with Odyssey[®] IRDye[®] 700 infrared dye labeled double-stranded oligonucleotides coupled with the EMSA buffer kit (LI-COR Bioscience, Lincoln, NE USA) according to manufacturer's instructions. In brief, 5 μ g of nuclear extract was incubated with 1 μ l of IRDye[®] 700 Infrared dye labeled double-stranded oligonucleotides, 2 μ l of 10 x binding buffer, 2.5 mM DTT, 0.25% Tween-20, and 1 μ g of poly (dI-dC) in a total volume of 20 μ l for 20 min at room temperature in the dark. Samples were separated on a 7% polyacrylamide gel in Tris-borate-EDTA running buffer for 40 min at 100 V. The gel was scanned by direct infrared fluorescence detection on the Odyssey[®] Imaging

System (LI-COR Bioscience, Lincoln, NE USA). NF- κ B IRDye[®] 700 infrared dye labeled oligonucleotide's sequences of the double-stranded DNA probes used were as follows: 5'-AGTTGAGGGGACTTTCCCAGGC-3' and 3'-TCAACTCCCCTGAAAGGGTCCG-5'. AP-1 IRDye[®] 700 infrared dye labeled oligonucleotide's sequences of the double-stranded DNA probes used were as follows: 5'-CGCTTGATGACTCAGCCGGAA-3' and 3'-GCGAACTACTGAGTCGGCCTT-5'. The underlined sequences in these oligonucleotide probes represent the binding site for each transcription factor. The specificity of the binding was examined using competition experiments, where 50~60-fold excess of the unlabeled oligonucleotides were added to the reaction mixture before adding the infrared dye labeled oligonucleotides.

Statistical analysis

All the experiments were repeated at least three times. Data were expressed as means \pm standard deviation (S.D.), and data were analyzed by paired Student's *t*-test to evaluate significant differences. A level of $p < 0.05$ was considered statistically significant.

Results

Cytotoxic effects of ascophyllan and fucoidans on RAW264.7 cells

Since it has been reported that fucoidan isolated from *Fucus vesiculosus* inhibits cell proliferation and induces apoptotic related signal pathway in human cancer cell lines and leukemic cell lines [23, 24], the cytotoxicities of ascophyllan, A-fucoidan, and S-fucoidan on RAW264.7 cells were examined. After 24 h incubation with varying concentrations of the polysaccharide samples, the viabilities of the cells were determined by the WST-8 assay. As shown in Fig. 1, ascophyllan and A-fucoidan showed no significant effects on RAW264.7 cells up to 1000 µg/ml, while S-fucoidan showed cytotoxic effect on RAW264.7 cells in a concentration-dependent manner.

NO levels in the culture supernatants of ascophyllan- and fucoidans-treated RAW264.7 cells

To examine whether or not ascophyllan, A-fucoidan or S-fucoidan can induce NO production in RAW264.7 cells, the cells were incubated with these polysaccharides at different concentrations (from 0 to 200 µg/ml). After 18 h incubation, the nitrite levels in the supernatants of the treated cells were examined by Griess assay. As shown in Fig. 2A, all these polysaccharides induced NO production from RAW264.7 cells, but the activities were quite different depending on the polysaccharide samples. The activity of ascophyllan was significantly stronger than those of fucoidans, while the activity of A-fucoidan was much lower than that of S-fucoidan. We also conducted a time-course analysis for the NO-production in RAW264.7 cells treated with each polysaccharide. As shown in Fig. 2B, 100 µg/ml of ascophyllan and S-fucoidan initiated to induce NO production after 8 h incubation from RAW264.7 cells, while the NO level in RAW264.7 cells treated with A-fucoidan was still quite low even after 24 h incubation.

Effects of ascophyllan and fucoidans on iNOS expression in RAW264.7 cells

As shown in Fig. 3, RT-PCR and western blot analysis revealed that significant levels of iNOS mRNA and protein were expressed in ascophyllan-treated RAW264.7 cells, which were evidently greater than those induced by S-fucoidan. However, the expression levels of iNOS mRNA and protein in RAW264.7 cells treated with A-fucoidan was almost trace levels.

TNF- α and G-CSF levels in RAW264.7 cells treated with ascophyllan and fucoidans

Previous studies have demonstrated that ascophyllan isolated from *A. nodosum* and fucoidan isolated from *Laminaria angustata* var. *longissima* induce the secretion of cytokines such as TNF- α from RAW264.7 cells [28, 41]. Consistent with previous findings, ascophyllan and fucoidans induced the secretion of TNF- α and G-CSF from RAW264.7 cells in a concentration-dependent manner. The higher activity of ascophyllan than those of fucoidans were observed especially at lower concentration-range (Fig. 4). Even at lower concentration range (3.1~25 $\mu\text{g/ml}$), significant levels of TNF- α and G-CSF were induced by ascophyllan, whereas these cytokines were undetectable in RAW264.7 cells treated with fucoidans at this concentration range. At 200 $\mu\text{g/ml}$, both S-fucoidan and ascophyllan induced almost equal levels of the cytokines, although the activity of A-fucoidan was still quite low.

Effects of ascophyllan and fucoidans on the activation of AP-1 and NF- κB in RAW264.7 cells

Therefore, an electrophoretic mobility shift assay (EMSA) on the nuclear extracts from ascophyllan or fucoidans-stimulated RAW264.7 cells was performed using IRD[®] 700 Infrared dye labeled oligonucleotide with NF- κB or AP-1 consensus sequence to analyze

the effects of three polysaccharides on the activation of NF- κ B and AP-1. As shown in Fig. 5, increases in the intensity of the bands reflecting the formation of complex with each transcription factor were observed in ascophyllan-treated RAW264.7 cells. The intensities of the bands with the equivalent positions in RAW264.7 cells treated with fucoidans were lower than those induced by ascophyllan or even not so clear. These results suggest that ascophyllan is capable of activating both NF- κ B and AP-1, and the activity is much greater than those of fucoidans. In the presence of excess amount of unlabeled oligonucleotide with the same sequence, the band corresponding to the complex with NF- κ B or AP-1 disappeared, suggesting the complex formation is specific. The activation of NF- κ B proceeds through phosphorylation and degradation of the I κ B- α inhibitory subunit and subsequent translocation of p65/p50 complex into the nucleus (42). Thus, nuclear translocation level of p65 in ascophyllan-treated RAW264.7 cells was also examined by immunoblotting. As shown in Fig. 6A, the nuclear level of the p65 protein increased in ascophyllan-treated RAW264.7 cells with greater extent than those induced by fucoidans. In addition, immunoblot analysis using specific antibodies indicated that both the phosphorylation and degradation of I κ B- α were also induced by ascophyllan with higher extent than those of fucoidans (Fig. 6B). These results suggest that ascophyllan strongly induces the nuclear translocation of NF- κ B through enhancing the phosphorylation and subsequent degradation of I κ B- α , and such activity of ascophyllan is stronger than fucoidans.

Effects of three MAP kinase inhibitors on NO-inducing activities of ascophyllan and fucoidans in RAW264.7 cells

MAP kinase signal pathways play an important role in NO-production in activated macrophages. To investigate whether or not MAP kinase signal pathways are involved in NO-production in RAW264.7 cells stimulated by ascophyllan and fucoidans, the effects of PD98059, SB202190 and SP600125, which are specific inhibitors for ERK, p38, and

JNK MAP kinase, respectively, were examined at final 10 μ M of each inhibitor and total 24 h incubation. Under the conditions, the inhibitors themselves showed no cytotoxic effect on RAW264.7 cells [39]. As shown in Table 1, SP600125 showed a potent inhibitory effect on ascophyllan-induced NO production in RAW264.7 cells, while PD98059 and SB202190 were less effective. Although the levels of NO induced by fucoidans were basically fairly low, PD98059 and SP600125 further decreased the NO levels, while SB202190 showed only partial inhibitory effect. These results suggest that JNK MAP kinase signal pathway may be mainly involved in NO production induced by ascophyllan in RAW264.7 cells. The different inhibition profiles with three MAP kinase inhibitors among ascophyllan and fucoidans may also suggest that MAP kinase system involved in the signaling pathway leading to NO production might not be necessarily the same between ascophyllan and fucoidans.

Effects of ascophyllan and fucoidans on three MAP kinases in RAW264.7 cells

To further investigate the involvement of MAP kinase signalling pathways in NO-inducing activities of ascophyllan and fucoidans, RAW264.7 cells were treated with each polysaccharide and the time-course analysis of the levels of phosphorylated and non-phosphorylated ERK, p38, and JNK MAP kinases were performed by western blotting. As shown in Fig. 7, increase in the phosphorylation levels of three MAP kinases were induced by ascophyllan and fucoidans with different time schedule and extent depending on the polysaccharide samples, and the activation levels of JNK and p38 after 60 min in ascophyllan-treated cells were evidently greater than fucoidans, while the phosphorylation levels of ERK MAP kinase were not much different between ascophyllan and fucoidans.

Effects of ascophyllan and fucoidans on mouse peritoneal macrophages

In addition to RAW264.7 cells, the effects of ascophyllan and fucoidans on mouse primary peritoneal macrophages were examined in terms of induction of NO and cytokines. As shown in Fig. 8, ascophyllan induced the production of NO, TNF- α , and G-CSF by peritoneal macrophages in a concentration-dependent manner, and the activities were higher than those of fucoidans.

Discussion

Ascophyllan is a sulfated polysaccharide isolated from the brown alga, *A. nodosum*. Ascophyllan mainly consists of L-fucose, xylose, uronic acid, and sulfated half-ester in approximately equimolecular proportion, and the composition is obviously distinct from those of A-fucoidan isolated from *A. nodosum* and S-fucoidan isolated from *Fucus vesiculosus* [28]. As characteristic composition features, ascophyllan has much high levels of uronic acid and xylose than those of fucoidans. Based on the composition analysis, ascophyllan is suggested to be a heteropolysaccharide (xylofucoglycuronan).

Regarding the biological activities of ascophyllan, our previous studies demonstrated that ascophyllan induced apoptotic cell death in U937 cells. We also found that ascophyllan induced the secretion of TNF- α and G-CSF from RAW264.7 cells. More recent studies have demonstrated that ascophyllan has growth-promoting activity on MDCK cells, while A-fucoidan was rather cytotoxic to this cell line, suggesting that ascophyllan is a quite distinct sulfated polysaccharide from A-fucoidan in terms of the biological activity as well [27, 28].

In the present studies, we found that ascophyllan induced NO production from RAW264.7 cells in a concentration-dependant manner at the concentration range tested (from 3.1 to 200 $\mu\text{g/ml}$). Interestingly, the activity of ascophyllan was much higher than those of fucoidans derived from *A. nodosum* (A-fucoidan) and *F. vesiculosus* (S-fucoidan). It is known that inducible NO synthase (iNOS) is a main enzyme responsible for NO production in activated macrophages [43]. Furthermore, it has been reported that S-fucoidan induced NO production from RAW264.7 cells by up-regulating the expression of iNOS mRNA and iNOS protein [33]. Thus, we analyzed the expression levels of iNOS in RAW264.7 cells treated with ascophyllan and fucoidans. Consistent with the increase in NO levels, significantly increased iNOS mRNA level and hence increased iNOS protein level were observed in ascophyllan-treated RAW264.7 cells, whereas the iNOS mRNA and protein levels induced by fucoidans were evidently lower

than those of ascophyllan or even at trace levels. These results strongly suggest that ascophyllan is a potent macrophage activator with higher activity at least than those of fucoidans tested. To further confirm this point, we compared the cytokine-inducing activities of ascophyllan and fucoidans. Although both ascophyllan and S-fucoidan induced the secretion of TNF- α and G-CSF from RAW264.7 cells in concentration-dependent manner, the activities of ascophyllan were evidently higher than those of S-fucoidan especially at lower concentration range (3.1~25 μ g/ml), at which no significant secretion of the cytokines were induced by S-fucoidan. A-Fucoidan showed even lower activities than those of S-fucoidan over the concentrations tested. These results further support the notion that ascophyllan is a potent macrophage activator. The reason for the differences in the activities to induce NO and cytokine production between S-fucoidan and A-fucoidan is unclear now, but we ascribe to the differences in the brown algae as the source of fucoidan. Because fucoidans have highly complicated and heterogeneous structures, their structures may vary depending on the algal species [44]. Cumashi *et al* investigated the anti-inflammatory, anti-angiogenic, anticoagulant and anti-adhesive properties of fucoidans obtained from nine species of brown algae. They found that the origin and composition of fucoidans could affect the biological activity of the fucoidans being tested to a certain extent [20]. The observed cytotoxic effect of S-fucoidan on RAW264.7 cells may also reflect the specific structural aspect of S-fucoidan that may be lack in ascophyllan and A-fucoidan.

NF- κ B and AP-1 are transcription factors regulating many important biological and pathological processes [45], and they are key transcription factors that modulate expression of various genes involved in immune and inflammatory responses, including iNOS and TNF- α [46-50]. In fact, the iNOS gene promoter contains several homologous consensus sequences for the binding of transcription factors such as NF- κ B, AP-1, and C/EBP [49, 50]. NF- κ B and AP-1 are believed to be essential for iNOS transcription [51, 52]. Thus, we investigated the possible involvement of NF- κ B and AP-1 in the induction of NO production by ascophyllan. To examine whether or not the activation of NF- κ B or

AP-1 occurs in ascophyllan-treated RAW264.7 cells, we carried out EMSA. The results shown in Fig. 5 suggested that NF- κ B and AP-1 were activated in ascophyllan-treated RAW264.7 cells, and the extent of the activation of these transcription factors were much significant than those of fucoidans. These results suggest that the stronger activity of ascophyllan to activate NF- κ B and AP-1 may partly explain the potent activity of ascophyllan to induce iNOS expression and NO production. Furthermore, the immunoblot analysis using the specific antibodies demonstrated that the NF- κ B activation processes such as phosphorylation and degradation of I κ B- α inhibitory subunit, and nuclear migration of p65 subunit were induced by ascophyllan with higher extent than those of fucoidans (Fig. 7), suggesting that ascophyllan has stronger activity to activate NF- κ B than fucoidans.

In addition to the transcription factors, it has been considered that many pathways are implicated in transmitting extracellular signals to the nuclei for iNOS gene expression [53]. In fact, it has been demonstrated that fucoidan induces protein tyrosine phosphorylation of protein kinases, protein kinase C activity, and specifically stimulates the activity of p21 activated kinase, ERK, JNK, and p38 MAPK leading to inflammatory cytokine secretion and urokinase-type plasminogen activator expression [54, 55]. Based on these findings, we investigated the effects of three different MAPK inhibitors, PD98059, SB202190 and SP600125, which are specific inhibitors for ERK, p38, and JNK MAP kinase, respectively. The results suggested that JNK and p38 but not ERK might play an important role in NO production in ascophyllan-treated RAW264.7 cells. Western blot analysis using specific antibodies against phosphorylated MAP kinases also suggested that the activation of three MAP kinases occurred in ascophyllan- or fucoidans-treated RAW264.7 cells with different extent. Especially the phosphorylation levels of JNK and p38 MAP kinases induced by ascophyllan were higher than those by fucoidans, while the levels of ERK MAP kinase were not much different among the polysaccharide samples (Fig. 7). Although further studies are required to clarify the exact way of the involvement of MAP kinase system in the ascophyllan-induced macrophage

activation, the results suggest that the potent activation of MAP kinases by ascophyllan may be partly explain the superior activity to induce NO and cytokine productions.

It has been reported that microalgal sulfated exopolysaccharide (MSE) from marine microalgal *Gyrodinium impudicum* induces NO production and tumoricidal activity of murine peritoneal macrophages [56]. Since the MSE-induced tumoricidal activity of macrophages was partly abrogated by a NO inhibitor, whereas the anti-TNF- α and anti-IFN- α/β antibodies as well as the scavengers of reactive oxygen intermediates had no effect, it was suggested that the tumoricidal activity of macrophages induced by MSE is mainly due to NO production [56]. In addition, the analysis using various inhibitors and EMSA on the intracellular signal transduction pathways leading to NO production in MSE-stimulated macrophages indicated that the activation of macrophages by MSE is mediated via the NF- κ B and JNK pathway [56]. Although the detailed entire structure of MSE is still unclear, and the origins of the polysaccharides are different, it seems likely that there is a similarity between MSE and ascophyllan regarding the involvement of JNK and NF- κ B in the NO production. Further studies are required to investigate the biological significance of the potent activity of ascophyllan to induce NO production from macrophages. Probably, studies on antitumor activities of ascophyllan in both *in vitro* and *in vivo* systems may provide us more useful information for practical application usage of ascophyllan. In our recent preliminary study, we have found that ascophyllan showed antitumor activity in Sarcoma-180 bearing mice model [57].

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Table 1 Effects of MAP kinase inhibitors on the ascophyllan- and fucoidans-induced NO production from RAW264.7 cells ^a

Inhibitors (10 μ M)	Ascophyllan	A-fucoidan	S-fucoidan
	NO levels (% of control) ^b		
+ PD98059	102.28 \pm 2.24%	61.59 \pm 8.43% *	19.81 \pm 12.76% *
+ SB202190	86.27 \pm 0.69% *	88.05 \pm 6.17%	96.79 \pm 2.43%
+ SP600125	44.01 \pm 2.58% *	47.46 \pm 0.76% *	22.32 \pm 4.97% *

^aAdherent RAW264.7 cells (3×10^4 cells/well in 96-well plates) were pre-incubated with each MAP kinase inhibitor (final 10 μ M) for 1 h at 37°C in the growth medium, and then ascophyllan, A-fucoidan, or S-fucoidan (final 200 μ g/ml) were added to the cells. After 24 h incubation, the nitrite levels in the culture medium from the treated cells were measured by Griess assay as described in the text.

^b The actual values of NO induced by ascophyllan, A-fucoidan, and S-fucoidan were 42.75 ± 1.79 , 2.80 ± 0.31 , and 6.11 ± 0.20 μ M, respectively. Data represents mean \pm S.D.. Asterisks indicate significant differences between with and without MAP kinase inhibitors ($p < 0.05$).

Figure legends

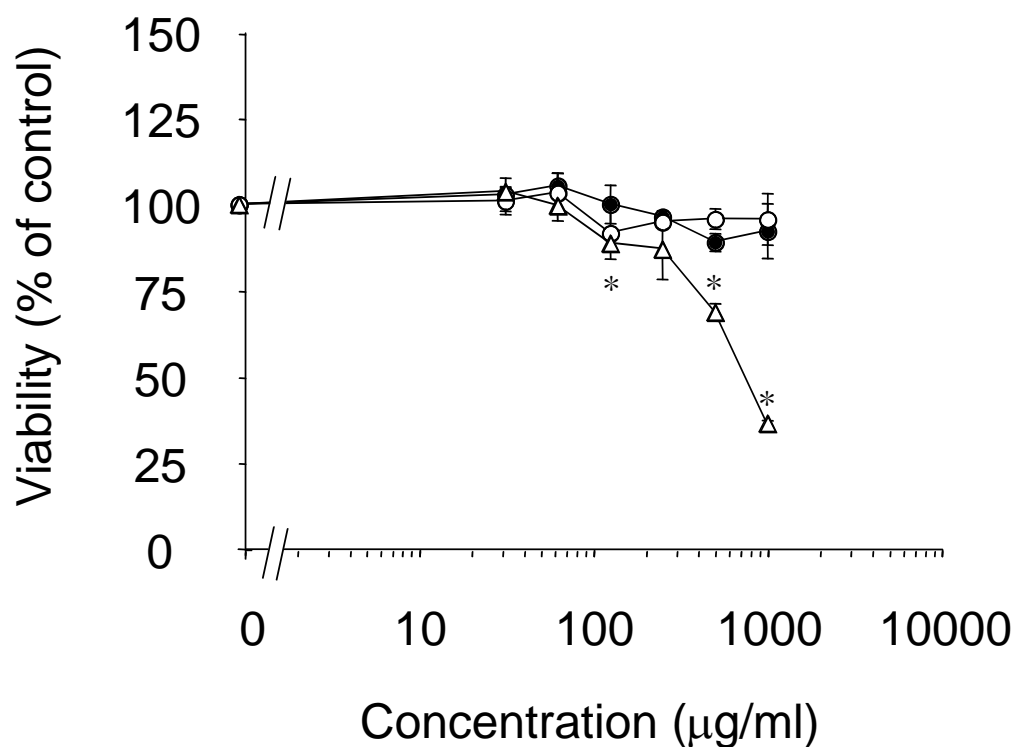


Fig. 1. Cytotoxicities of ascophyllan and fucoidans on RAW264.7 cells. Adherent RAW264.7 cells (3×10^4 cells/well in 96-well plates) were incubated with varying concentrations of ascophyllan (●), A-fucoidan (○), or S-fucoidan (△) at 37°C in the growth medium. After 24 h incubation, the viabilities of the treated cells were examined by WST-8 assay as described in the text. Data represents average of triplicate measurements and bars indicate the standard deviations. Asterisks indicate significant differences between with and without polysaccharides ($p < 0.05$).

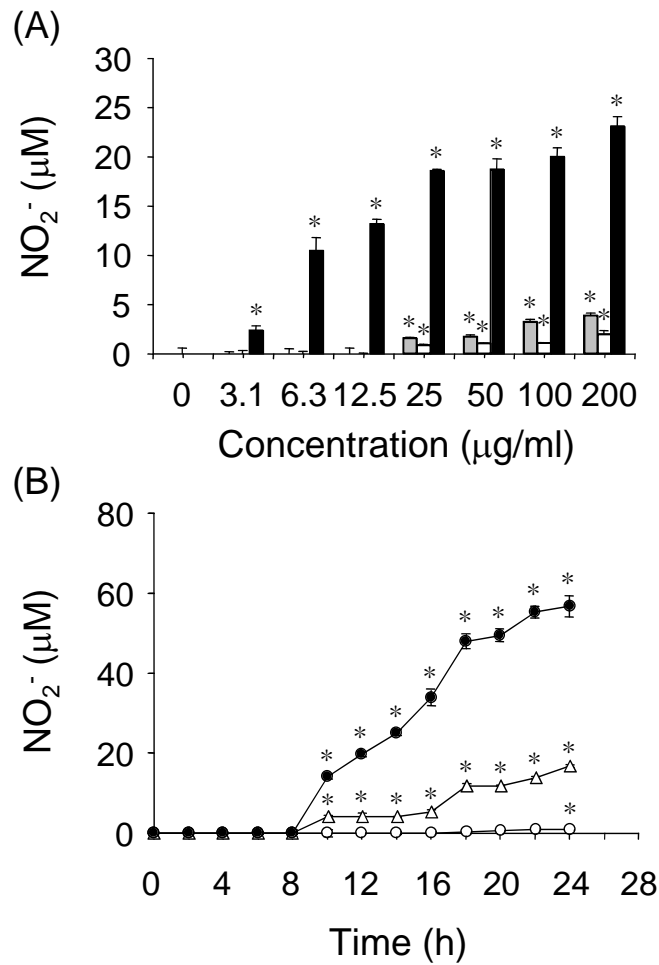


Fig. 2. Nitric oxide (NO) levels in RAW264.7 cells treated with ascophyllan and fucoidans. (A) Adherent RAW264.7 cells (3×10^4 cells/well in 96-well plates) were incubated with varying concentrations of ascophyllan (■), A-fucoidan (□), or S-fucoidan (■) at 37°C in the growth medium. After 18 h incubation, the nitrite levels in the culture medium from the treated cells were measured by Griess assay as described in the text. (B) For the time course analysis, adherent RAW264.7 cells (5×10^5 cells/well in 24-well plates) were incubated with ascophyllan (●), A-fucoidan (○), or S-fucoidan (△) at the concentration of 100 µg/ml in the growth medium at 37°C. The nitrite levels in the culture medium of the treated cells were measured 2 h interval by Griess assay as described in the text. Data represents average of triplicate measurements and bars indicate the standard deviations. Asterisks indicate significant differences between with and without polysaccharides ($p < 0.05$).

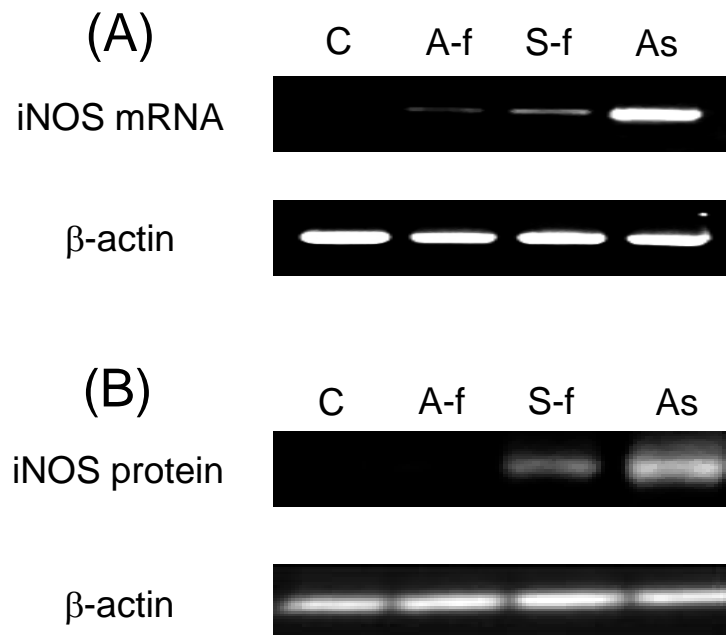


Fig. 3. iNOS mRNA and protein levels in RAW264.7 cells treated with ascophyllan and fucoidans. (A) Adherent RAW264.7 cells (5×10^5 cells/well in 24-well plates) were incubated with ascophyllan, A-fucoidan, or S-fucoidan at the concentration of $100 \mu\text{g/ml}$ in the growth medium at 37°C . After 4 h incubation, iNOS mRNA levels in RAW264.7 cells treated with ascophyllan, A-fucoidan, or S-fucoidan were analyzed by RT-PCR method as described in the text. (B) Adherent RAW264.7 cells (1×10^6 cells/well in 12-well plates) were incubated with polysaccharide samples at the concentration of $100 \mu\text{g/ml}$ in the growth medium at 37°C . After 6 h, the whole-cell lysates were prepared from the treated cells, and analyzed by western blot analysis as described in the text. Control; C, +A-fucoidan; A-f, +S-fucoidan; S-f, +ascophyllan; As.

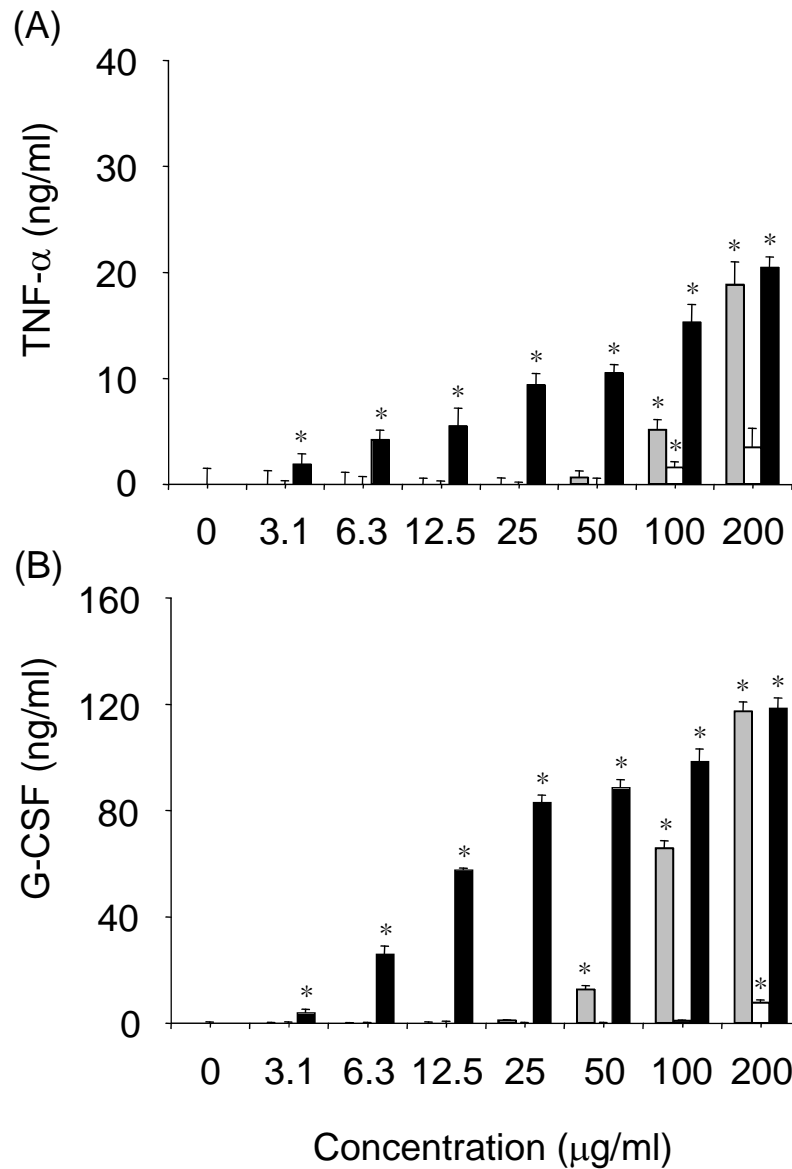


Fig. 4. TNF- α and G-CSF levels in RAW264.7 cells treated with ascophyllan and fucoidans. Adherent RAW264.7 cells (3×10^4 cells/well in 96-well plates) were incubated with varying concentrations of ascophyllan (■), A-fucoidan (□), or S-fucoidan (■) in the growth medium at 37°C. After 18 h incubation, the levels of TNF- α (A) and G-CSF (B) in the culture supernatants of the treated cells were measured by ELISA as described in the text. Data represents average of triplicate measurements and bars indicate the standard deviations. Asterisks indicate significant differences between with and without polysaccharides ($p < 0.05$).

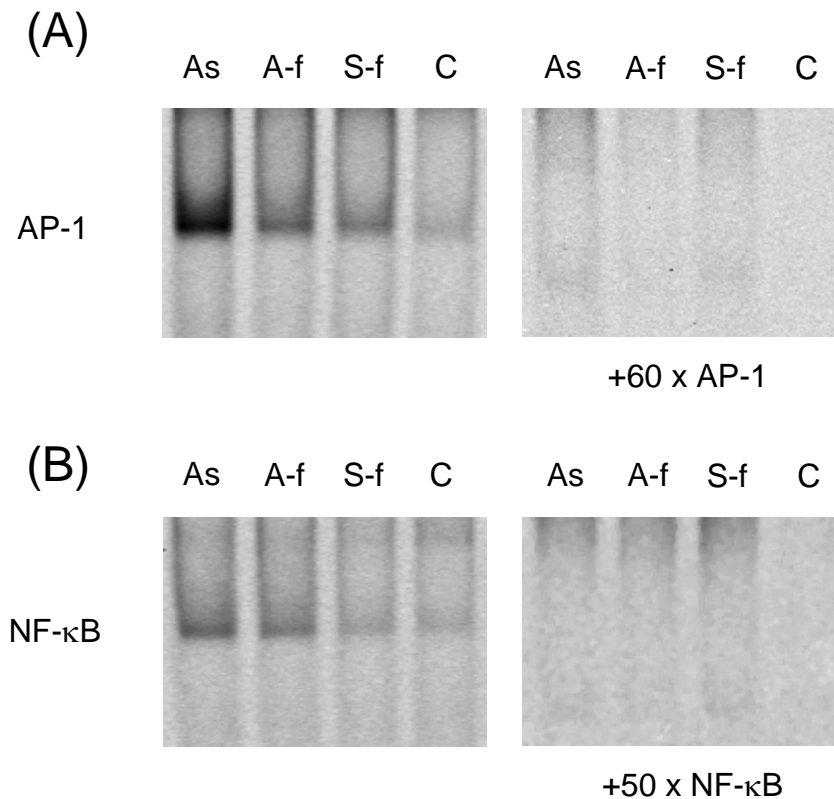


Fig. 5. Effects of ascophyllan and fucoidans on the activation of AP-1 and NF-κB in RAW264.7 cells. Adherent RAW264.7 cells (4×10^6 cells/3.5 cm dish) were incubated with 100 μg/ml of each polysaccharide sample in the serum-free DMEM at 37°C. The untreated cells were used as control. After 1 h incubation, the nuclear proteins were extracted from the treated cells, and were examined by EMSA in the absence or presence of 50~60-fold excess unlabeled oligonucleotides with the same NF-κB or AP-1 consensus sequence for the binding with an oligonucleotide probe corresponding to a consensus AP-1- (A) or NF-κB- (B) binding sequence as described in the text. The detection and analysis of the complexes were performed with Odyssey[®] IRD[®] 700 infrared dye detection system according to the manufacturer's instructions. Control; C, +A-fucoidan; A-f, +S-fucoidan; S-f, +ascophyllan; As.

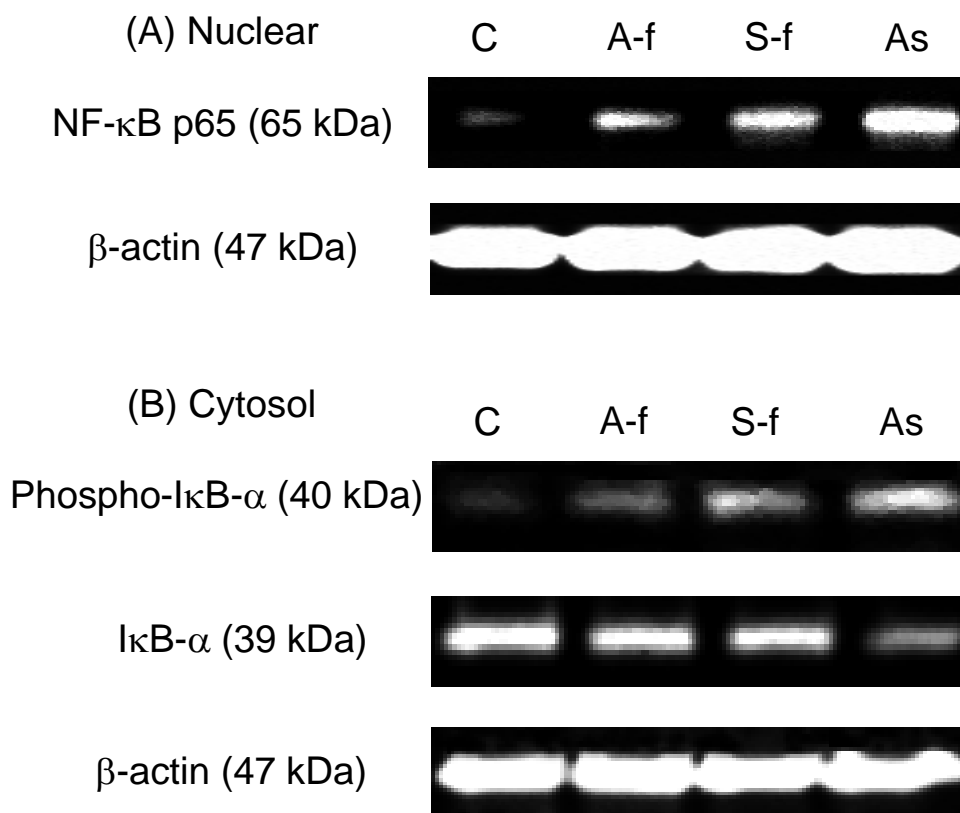


Fig. 6. Effects of ascophyllan and fucoidans on the nuclear translocation of NF- κ B p65, and phosphorylation and degradation of I κ B- α in RAW264.7 cells. (A) Western blot analysis was conducted on NF- κ B p65 in the nuclear extracts prepared from RAW264.7 cells treated with 100 μ g/ml of each polysaccharide sample as described in the text. (B) Western blot analysis was conducted on the I κ B- α and phosphorylated I κ B- α in the cytosolic extracts prepared from RAW264.7 cells treated as described above.

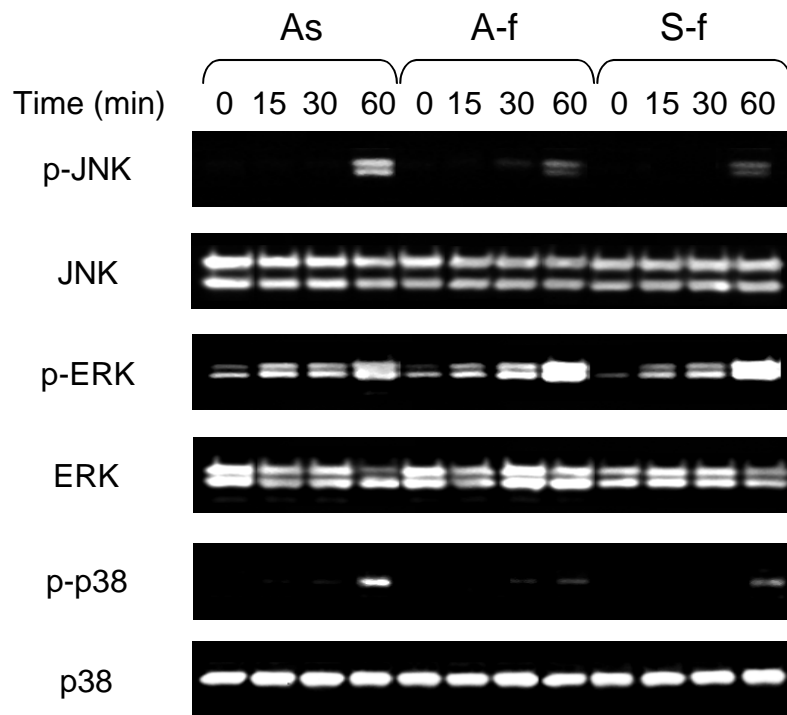


Fig. 7. Effects of ascophyllan and fucoidans on MAP kinases in RAW264.7 cells. Adherent RAW264.7 cells (4×10^6 cells/3.5 cm dishes) were incubated with polysaccharide samples (final 100 $\mu\text{g/ml}$) in serum-free DMEM at 37°C . The whole-cell lysates were prepared from the treated cells after the indicated periods of time, and the levels of both phosphorylated and nonphosphorylated MAP kinases were analyzed by western blotting as described in the text.

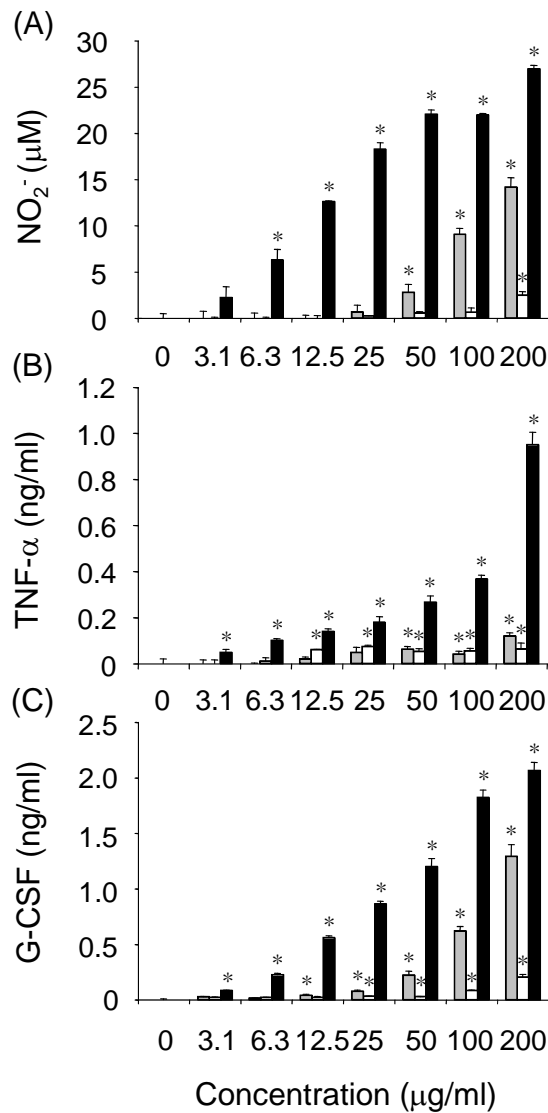


Fig. 8. (A) NO, (B) TNF- α , and (C) G-CSF levels in mouse peritoneal macrophages treated with ascophyllan and fucoidans. Adherent macrophage (3.5×10^4 cells/well in 96-well plates) were incubated with varying concentrations of ascophyllan (■), A-fucoidan (□), or S-fucoidan (■) at 37°C in the growth medium. After 18 h incubation, the levels of NO and cytokines (TNF- α , and G-CSF) in the culture medium were measured by Griess assay and ELISA, respectively as described in the text. Data represents average of triplicate measurements and bars indicate the standard deviations. Asterisks indicate significant differences between with and without polysaccharides ($p < 0.05$).