Inhibitory Effect of Orally-administered Sulfated Polysaccharide Ascophyllan Isolated from *Ascophyllum nodosum* on the Growth of Sarcoma-180 Solid Tumor in Mice

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Abstract. *We evaluated the antitumor activity of crude extract and ascophyllan prepared from Ascophyllum nodosum in sarcoma-180 solid tumor-bearing mice with continuous intraperitoneal (i.p.) administration at a dose of 50 mg/kg body weight/day or oral administration at a dose of 500 mg/kg body weight/day. Ascophyllan and crude extract administered via the oral route showed greater antitumor effects than via i.p. route, and the tumor sizes in mice treated with ascopyllan and crude extract were reduced by a mean of 68.7±6.8% and 42.4±24.8% by the oral route, and 41.4±16.1% and 13.6±20.6% by i.p. route compared to control mice. Splenic natural killer cell activity in the mice treated with ascophyllan and crude extract by i.p. route was significantly enhanced, while only a slight increase of this activity was observed in orally-treated mice. Furthermore, increase in spleen weight of tumor-bearing mice was slightly suppressed by oral administration of ascophyllan, whereas i.p. administration resulted in further enlargement. Analysis of serum cytokines revealed that oral treatment with ascophyllan resulted in significant increase of tumor necrosis factor-α and interleukin-12 levels. Since ascophyllan showed no direct cytotoxic effect on sarcoma-180 cells, orally-administered ascophyllan is suggested to exhibit its antitumor activity through the activation of the host immune system.*

Cancer is one of the most serious diseases for human beings. Although the efficacy of modern medical treatments for cancer has improved over recent years, the associated side-effects still cause serious problems limiting the rehabilitation

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and the survival rate of patients (1, 2). Therefore, further efforts are required for discovery of new anticancer strategies such as utilization of natural products as medicines for a safe and feasible approach to cancer treatment.

Certain seaweeds have long been used in traditional Chinese medicine for the cure of malignant tumors (3), and consumption of brown algae and their extracts may contribute to reducing the incidence of cancer, especially of breast cancer in Asian countries (4). Hence, much attention has been focused on natural compounds isolated from brown algae. Yamamoto *et al.* reported that the non-dialyzable fractions consisting of polysaccharides from four brown seaweeds markedly inhibited sarcoma-180 solid tumor growth in mice (5, 6). They further reported that the main antitumor components against L-1210 leukemia in a mouse model were sulfated polysaccharides (7, 8). Similarly, crude fucoidan from *Eisenia bicyclis* was been found to exert immune-potentiating effects in sarcoma-180 tumor-bearing mice, leading to antitumor effects (9). Furthermore, fucoidan purified from brown seaweed *Sargassum thunbergii* was reported to inhibit the growth of Ehrlich ascites carcinoma and the metastasis of Lewis lung carcinoma in mice through enhancement of host-immune responses (10, 11). It has recently been reported that fucoidan isolated from *Fucus vesiculosus* showed antitumor activities against sarcoma-180, Lewis lung carcinoma and B16 melanoma in mouse models. It was proposed that the anti-angiogenic activity of the fucoidan is partly responsible for the antitumor mechanisms, and the sulfation level in the fucoidan molecule plays an important role in the antiangiogenic and antitumor activities (12).

Ascophyllan, a sulfated polysaccharide isolated from brown alga *Ascophyllum nodosum*, is distinguishable from fucoidan in terms of its characteristic monosaccharide composition (13) and biological activity (14, 15). Interestingly, ascophyllan induced production of much higher levels of nitric oxide (NO) and cytokines, such as tumor necrosis factor-α (TNF-α) and granulocyte colony-stimulating factor (G-CSF), from RAW264.7 cells than those

induced by fucoidans isolated from *F. vesiculosus* and *A. nodosum* (15). A more recent study found that ascophyllan was capable of stimulating a respiratory burst in RAW264.7 cells (16). Furthermore, our previous studies found that intraperitoneally-administered ascophyllan showed antitumor activity in a sarcoma-180 ascites tumor model (17). Similar to fucoidan (18), we have recently found that ascophyllan administered by intraperitoneal (*i.p.*) injection increased the natural killer (NK) activity of splenic lymphocytes (19). Thus, ascophyllan is considered to be a promising candidate as a cancer chemotherapeutic agent.

In the present study, we evaluated the antitumor activities of purified ascophyllan together with crude extract of *A. nodosum* in sarcoma-180 solid tumor-bearing mice through *i.p.* and oral administration routes.

Materials and Methods

Materials. Calcein-AM was obtained from Molecular Probes, Inc. (Eugene, OR, USA). Mouse TNF-α enzyme-linked immunosorbent assay (ELISA) kit and mouse interleukin-12 (IL-12) total ELISA kit were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). ELISA kit to mouse interferon-γ (IFN-γ) was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Other chemicals were of the highest grade commercially available.

Preparation of crude extract and ascophyllan. Brown seaweed *A. nodosum* collected on the coast of Norway was obtained from KAISEI (Shimonoseki, Japan). Ascophyllan was prepared from *A. nodosum* as described previously (13). Crude extract was prepared by the following procedures. Milled *A. nodosum* was suspended in water and stirred at 100˚C for 1 h. The water extraction was repeated twice. After filtration, activated charcoal was added to the filtrate and stirred at 100˚C for 30 min. After removal of activated charcoal by filtration, the solution was subjected to spray drying, and the obtained powder was used as a crude extract. The crude extract is currently commercially available as Ascophyllan HS from Hayashikane Sangyo Co. (Yamaguchi, Japan). Ascophyllan and crude extract solutions were passed through endotoxin-removal filters (Zetapor Dispo: Wako Pure Chemical industries, Ltd, Osaka, Japan) before use.

Molecular mass and composition analysis. Molecular mass of ascophyllan was estimated by a gel-filtration chromatography with a Sepharose 4B column (1.5 cm × 100 cm) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) as described previously (20). Composition of neutral monosaccharide in ascophyllan was analyzed by Alliance® high-performance liquid chromatography (HPLC) system (Milford, MA, USA) equipped with GlyScope Honenpak C18 column (75 mm × 4.6 mm I.D.) (J-OIL MILLS, INC., Tokyo, Japan), as previously reported (20). Uronic acids in ascophyllan were estimated by a modified carbazole-sulfuric acid method using galacuronic acid as a standard (20). The sulfate level of ascophyllan was measured with Dodgson-Price method, using K_2SO_4 as a standard, as reported previously (20).

Animals and cell lines. Specific pathogen-free (SPF) male ddY mice (4 weeks old, 17.0-26.0 g) were obtained from Texam, Nagasaki, Japan. These mice were housed at a constant room temperature of 24˚C under controlled conditions of 12 h light/dark cycle photoperiod and provided with free access to standard laboratory food CE-2 (Texam, Nagasaki, Japan). All mice were treated according to the Guidelines of the Japanese Association for Laboratory Animal Science and the Guidelines for Animal Experiments of Nagasaki University, Japan. Murine sarcoma-180 cells obtained from the Institute of Development, Aging, and Cancer of Tohoku University were maintained in the abdominal cavity of 4-week-old SPF male ddY mice. A murine T-lymphoma cell line YAC-1, which is sensitive to the cytotoxic activities of naturally-occurring killer cells in mice, obtained from the Institute of Development, Aging, and Cancer of Tohoku University, were cultured at 37˚C in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (100 μg/ml) in a humidified atmosphere with 5% CO_2 and 95% air.

Cytotoxicity assay. Cytotoxic effects of ascophyllan and crude extract on sarcoma-180 cells were measured by the Alamar blue assay as described previously (14). Briefly, 10-day-old sarcoma-180 ascites cells, obtained from the abdominal cavities of ascites tumor-bearing mice, were washed twice with phosphate buffered saline (PBS) by centrifugation (270 ×*g*) for 5 min at 4˚C, and then seeded into 96-well plates (2×10^4 cells/well) with different concentrations of ascophyllan or crude extract (0-1,000 μg/ml by two-fold dilution) in RPMI-1640 medium supplemented with 20% FBS. After 48 h at 37˚C, Alamar blue reagent was added to each well at a final concentration of 10%. After 3 h incubation at 37˚C, the absorbance of each well was measured at 535 nm with reference wavelength at 600 nm using a Multiskan GO microplate reader (Thermo Fisher Scientific K.K., Yokohama, Japan).

In vivo antitumor assay. *In vivo* antitumor activities of ascophyllan and crude extract with different routes of continuous administration (*i.p.* or oral route) were evaluated in a sarcoma-180 solid tumor model as previously reported, with some modifications (21, 22). Ascophyllan and crude extract were dissolved in saline for *i.p.* or distilled water for oral administration. Administration of ascophyllan or crude extract *i.p.* (dose: 50 mg/kg b.w./day; n=10) and orally (dose: 500 mg/kg b.w./day; n=8) to mice were initiated two days and four days prior to tumor implantation, respectively. Twelve hours after final administration of each sample, 50 μl of 10-day-old sarcoma-180 ascites tumor cells (1×10^8 cells/ml) was intra-dermally inoculated into the dorsal area of test mice. Subsequently, ascophyllan or crude extract solution was consecutively administered to tumor-bearing mice *via i.p.* or oral route once daily for 10 consecutive days. Control tumor-bearing mice and normal mice were administered saline (for *i.p.* route; n=10) or distilled water (for oral route; n=8) on the same schedule. Mortalities and body weights of mice in each group were recorded every day from the beginning of ascophyllan and crude extract administration to sacrifice day. On day 11 after tumor transplantation, all mice were sacrificed, and then the solid tumor tissues were excised and weighed for evaluating the *in vivo* antitumor activity. The tumor volume was estimated according to the following formula: tumor volume (cm^3)=xyz, where x, y, and z are the three perpendicular diameters of the tumor. Tumor growth inhibition ratio was calculated by following formula: inhibition ratio (%)=$[(A-B)/A] \times 100$, where A is average tumor weight (W_C) or volume (V_C) of control tumor-bearing mice, and B is tumor weight (W_T) or volume (V_T) of mice treated with test sample (ascophyllan or crude extract). The major organs (liver, spleen, and kidney) were also excised and weighed at the same time.

Table I. *Chemical composition and molecular mass of ascophyllan isolated from Ascophyllum nodosum.*

| | SO$_4^{2-}$ (%)[a] | Neutral monosaccharides (%)[b] (Mol of L-fucose was considered as 1) | | | | | Uronic acid (%)[c] (Molar ratio) | Molecular mass[d] (kDa) |
		Fuc	Xyl	Gal	Man	Glc		
Ascophyllan	7.14	16.79 (1.00)	14.56 (0.95)	3.63 (0.20)	1.15 (0.06)	0. 004 (TL)	30.31 (1.53)	98

[a]Determined by turbidity assay after hydrolysis with 2 M of HCl; [b]Determined by high-performance liquid chromatography analysis after hydrolysis with 4 M of trifluoroacetic acid; [c]Determined by carbazole-sulfuric acid assay as glucuronic acid equivalent; [d]Determined by Sepharose 4B gel-filtration chromatography. TL, Trace level.

Splenic NK cell activity analysis. Three mice were selected randomly from each group, and splenic NK cell activity was measured by a method described previously (19). Briefly, 15 μM of calcein-AM was added to the YAC-1 cells (1.5×10^6 cells/ml) in RPMI-1640 medium supplemented with 10% FBS, and the cells were incubated for 30 min at 37°C with gentle stirring. The calcein-loaded YAC-1 cells were washed twice with the medium and resuspended in the medium containing 2.5 mM of probenecid, an anion transporter inhibitor. Two hundred microliters of the calcein-loaded YAC-1 cells (10^6 cell/ml) was added to 200 μl of splenic lymphocytes (5×10^7 cells/ml) prepared from mice of each group at effector/target ratio of 50:1. After incubation at 37°C for 4 h, the cells were centrifuged, then 40 μl of supernatant was harvested and mixed with 500 μl of 40 mM Tris-HCl (pH 8.0) buffer. The fluorescence intensity at 530 nm excited at 485 nm was then measured with a F-2500 fluorescence spectrophotomer (Hitachi High-Technologies Co., Tokyo, Japan)

Analysis of cytokines in the serum. Blood was collected from the portal vein of each mouse, and serum was obtained by centrifugation. The levels of TNF-α, IL-12, and IFN-γ in the serum of tumor-bearing mice and control mice were determined by sandwich ELISA with two antibodies to two different epitopes on TNF-α, IL-12, or IFN-γ molecule according to the instructions of the manufacturers. The concentration of each cytokine was estimated from a reference to a standard curve for serial three-fold dilution of murine recombinant TNF-α, IL-12, or IFN-γ. According to the manufacturers' instructions, the detection limit of ELISA kit for TNF-α, IL-12, and IFN-γ and 9 pg/ml, 12 pg/ml, and 2 pg/ml, respectively.

Statistical analysis. All experiments were repeated at least three times. The results are expressed as means±S.E., and the data were analyzed using one-way ANOVA followed by *t*-test to determine significant differences. A value of *p*<0.05 was considered statistically significant.

Results

Molecular mass and composition analysis of ascophyllan. Based on a gel-filtration profile, the mean molecular mass of ascophyllan was estimated to be 98 kDa (Table I). The monosaccharide composition analysis is shown in Table I. The composition values were consistent with the values previously reported (13). However, the mean molecular mass of ascophyllan (98 kDa) was lower than the value previously estimated (390 kDa) (20).
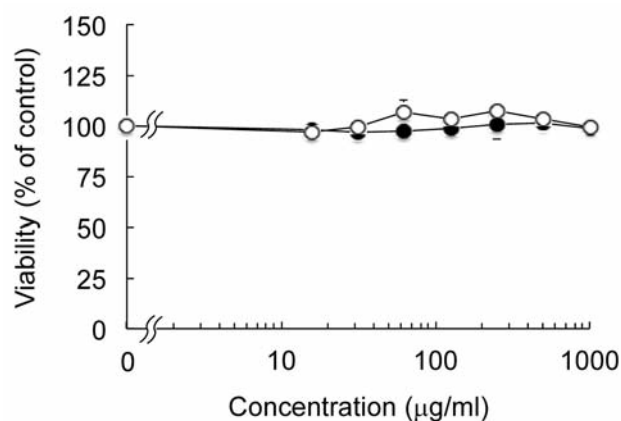

Figure 1. *Direct cytotoxic effect of ascophyllan and crude extract on sarcoma-180 cells. Ten-day-old sarcoma-180 ascites cells were harvested from sarcoma-180 ascites tumor-bearing mouse and washed twice with phosphate buffered saline (PBS) by centrifugation. The cells suspended in growth medium in 96-well plates (2×10^4 cells/well) were incubated with different concentrations of ascophyllan (●) or crude extract (○) (0-1,000 μg/ml) at 37°C for 48 h. The viability of the cells was measured by the Alamar blue assay as described in the text. Data represent means±S.E. (n=3).*

Direct cytotoxic effects of ascophyllan and crude extract on sarcoma-180 cells. As shown in Figure 1, both ascophyllan and crude extract showed no cytotoxic effects on sarcoma-180 cells up to a concentration of 1,000 μg/ml.

Antitumor activity of ascophyllan and crude extract. As shown in Figure 2, *i.p.* and orally-administered ascophyllan had significant inhibitory effects on sarcoma-180 solid tumor growth. As judged from tumor weights and volumes, significantly greater antitumor activity of orally-administered ascophyllan than that administered by *i.p.* route was observed. Tumor weights and volumes of mice treated with ascophyllan orally were reduced by 42.3±7.5% and 68.7±6.8% of those of control mice, respectively (Table II; Figure 2B), whereas reduction of tumor weights and volumes of the mice treated with ascophyllan *i.p.* were 25.8±26.8% and 41.4±16.1%, respectively (Table II; Figure 2A). Crude
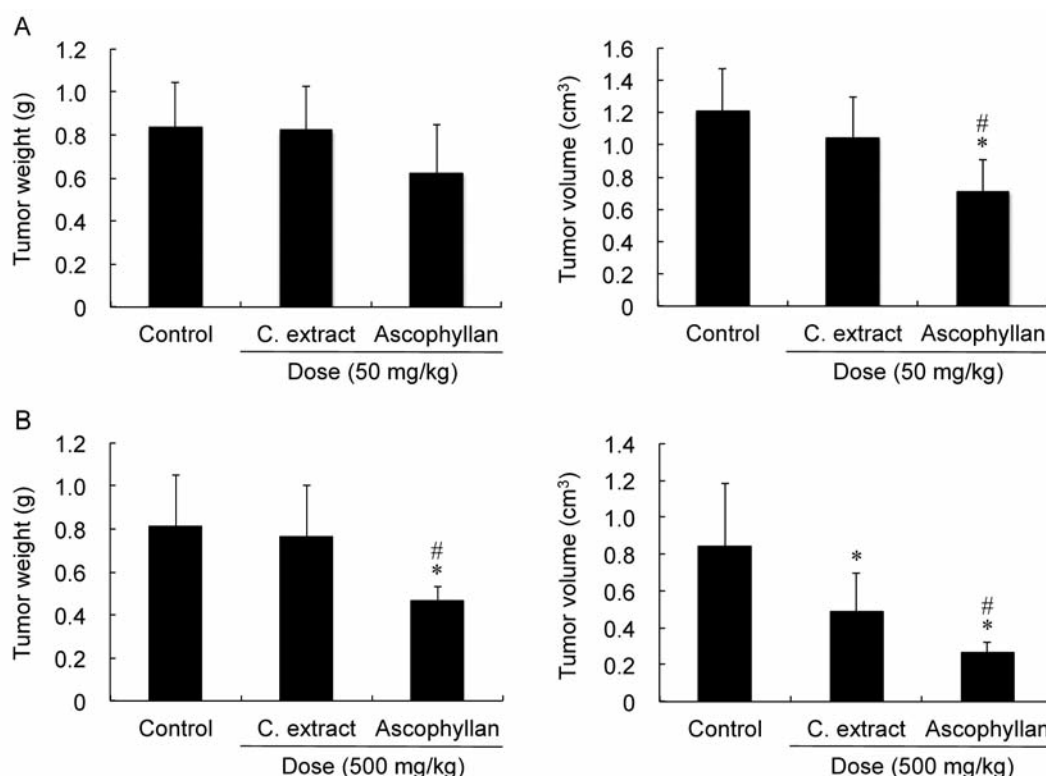
Figure 2. *Antitumor activity of ascophyllan and crude extract via continuous i.p. (A) or oral (B) administration to sarcoma-180 solid tumor-bearing mice. Average tumor weights and volumes are shown. Data represent means±S.E. (n=10 for i.p. administration, n=8 for oral administration). Significant difference between *treated and untreated control tumor-bearing mice, and #ascophyllan- and crude extract-treated tumor-bearing mice (p<0.05) were found.*

Table II. *Antitumor activity of ascophyllan and crude extract via continuous i.p. or oral administration in sarcoma-180 solid tumor-bearing mice.*

Administration route		Surviving mice	Inhibition ratio (%)	Inhibition ratio (%)
		Total mice	$[(W_C-W_T)/W_C]\times100$	$[(V_C-V_T)/V_C]\times100$
Ascophyllan	*i.p.*	7/10	25.8±26.8	41.4±16.1*
	Oral	8/8	42.3±7.5*	68.7±6.8*#
Crude extract	*i.p.*	6/10	1.9±24.4	13.6±20.6
	Oral	8/8	5.9±29.3	42.4±24.8*#

W_C and W_T indicate the average tumor weight of control tumor-bearing mice and the tumor weight of ascophyllan- or crude extract-treated tumor-bearing mice, respectively; V_C and V_T indicate the average tumor volume of control tumor-bearing mice and the tumor volume of ascophyllan- or crude extract-treated tumor-bearing mice, respectively. Data represent means±S.E. (n=10 for *i.p.* administration, n=8 for oral administration). Significant difference between *ascophyllan- or crude extract-treated tumor-bearing mice and control tumor-bearing mice, and #tumor-bearing mice treated *via* the oral route and the *i.p.* route (*p*<0.05) were found.

extract also showed antitumor activity but was less effective compared to ascophyllan. Slight but evidently better antitumor effect of crude extract with the oral rather than *i.p.* route was also observed (Table II; Figure 2). During the treatment with ascophyllan or crude extract, 30% or 40% of treated mice died in the *i.p.* administration groups, while no mice in the oral-administration groups died (Table II). These results suggest that the therapeutic efficacy of ascophyllan against sarcoma-180 solid tumor might differ depending on the administrative route, and that through the oral route may exert a greater effect than that through the *i.p.* one.

Effects of ascophyllan and crude extract on body and organ weights in tumor-bearing mice. Regarding the gains of body
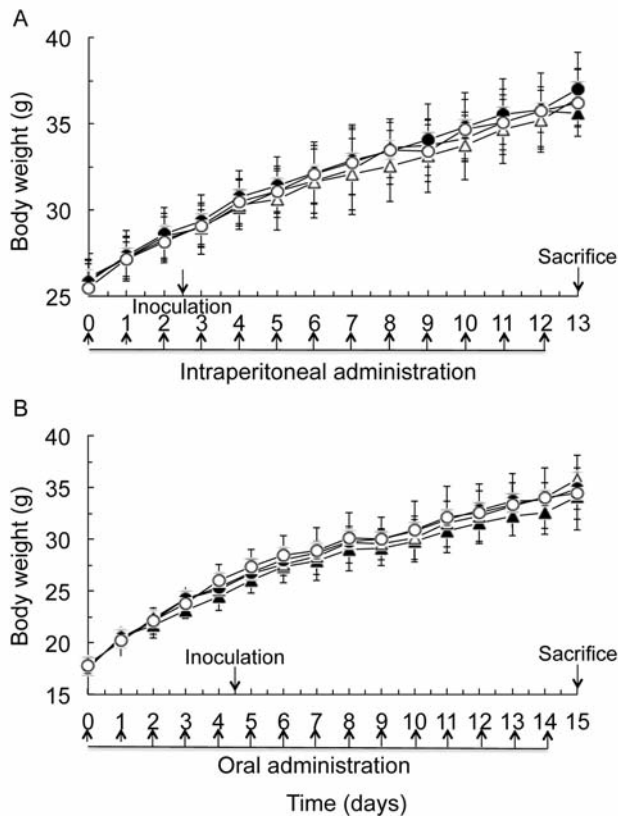
Figure 3. *Administration schedules of ascophyllan and crude extract, and their effects on the body weights of tumor-bearing mice. A, Ascophyllan (▲) or crude extract (△) were continuously administered to tumor-bearing mice via i.p. route at the dose of 50 mg/kg b.w./day once daily for 10 days. Saline was injected to control tumor-bearing mice (●) and normal mice (○) with the same schedule. B, Ascophyllan (▲) or crude extract (△) were continuously administered to tumor-bearing mice via oral route at the dose of 500 mg/kg b.w./day once daily for 10 days. Distilled water was orally administered to control tumor-bearing mice (●) and normal mice (○) with the same schedule. The body weights of mice from each group were recorded daily from the beginning of treatment to sacrifice. Data represent means±S.E. (n=10 for i.p. administration, n=8 for oral administration).*

Figure 4. *Effects of ascophyllan and crude extract on the spleen weights in tumor-bearing mice treated with continuous i.p. or oral administration. Average spleen weights in normal mice, control tumor-bearing mice, and tumor-bearing mice treated with continuous i.p. (A) or oral (B) administration of ascophyllan or crude extract. Data represent means±S.E. (n=10 for i.p. administration, n=8 for oral administration). Significant difference between *asphyllan-treated and control tumor-bearing mice, and #tumor-bearing mice and normal mice (p<0.05) were found.*

weight during *i.p.* and oral treatment with ascophyllan or crude extract, no significant differences between treated and normal mice were observed (Figure 3). Although the weights of liver and kidney of the treated mice were almost the same as those in normal mice (data not shown), the spleens of tumor-bearing mice were significantly enlarged compared to those of normal mice (Figure 4). Administration of ascophyllan *i.p.* resulted in a further increase of spleen weights (Figure 4A), whereas spleen weights of the mice administered with ascophyllan orally were slightly reduced (Figure 4B). Similar effects of crude extract on spleens were also observed, whilst these effects were weaker than those of ascophyllan (Figure 4).
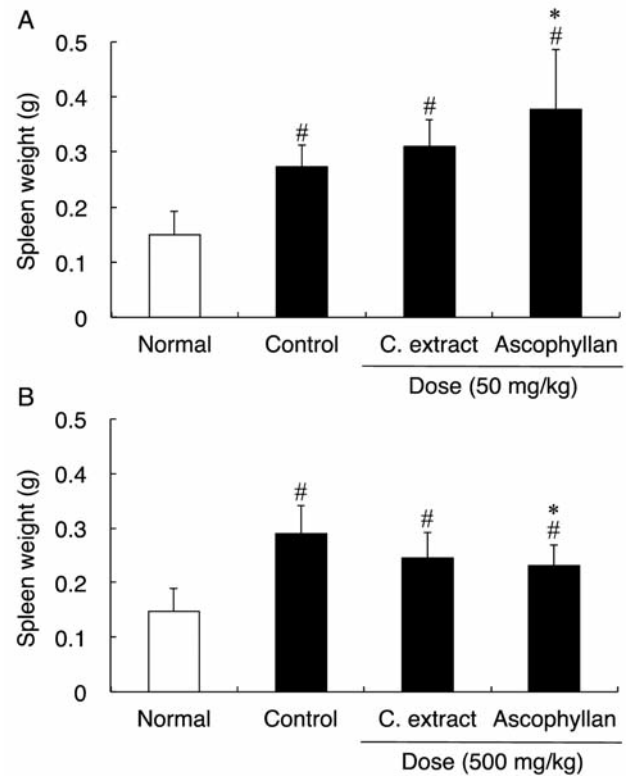
Effects of ascophyllan and crude extract on splenic NK cell activity in tumor-bearing mice. To investigate whether ascophyllan or crude extract affects the splenic NK cell activity of tumor-bearing mice, the cytotoxic effects of spleen cells of tumor-bearing mice treated with ascophyllan or crude extract against YAC-1 cells were examined. As shown in Figure 5, continuous *i.p.* administration of ascophyllan significantly enhanced the splenic NK cell activity in tumor-bearing mice compared to that of control tumor-bearing mice (Figure 5A), while only a slight increase in this activity was observed in tumor-bearing mice orally treated with ascophyllan (Figure 5B). On the other hand, no significant difference in splenic NK cell activity in the tumor-bearing mice treated with crude extract *via* either *i.p.* or the oral route was observed (Figure 5).

Effects of ascophyllan and crude extract on cytokine levels in tumor-bearing mice. To gain insight into the underlying mechanism of antitumor activity of ascophyllan and crude
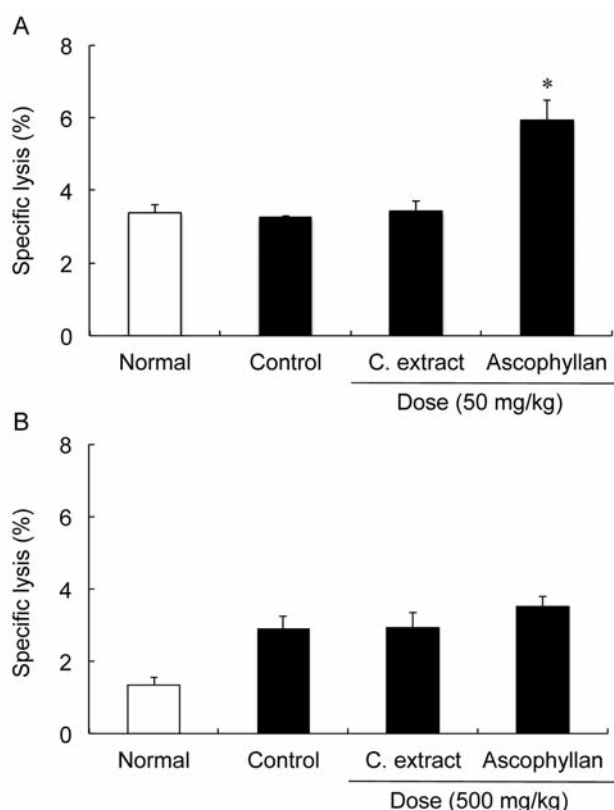
Figure 5. *Effects of ascophyllan and crude extract on the splenic natural killer (NK) cell activity in tumor-bearing mice treated with continuous i.p. (A) or oral (B) administration. Data represent means±S.E. (n=3 for i.p. administration, n=3 for oral administration). *Significant difference between ascophyllan-treated tumor-bearing mice and control tumor-bearing mice (p<0.05) were found.*

extract, the serum levels of TNF-α, IL-12, and IFN-γ in mice treated with these compounds were measured by sandwich ELISA. As shown in Figure 6, the serum levels of TNF-α and IL-12 in tumor-bearing mice treated with continuous *i.p.* or oral administration of ascophyllan were significantly increased compared to those in control tumor-bearing and normal mice. Continuous *i.p.* and oral administration of crude extract also induced increased levels of TNF-α and IL-12, while cytokine levels were lower than those induced by ascophyllan (Figure 6A, B, D and E). Intraperitoneally-administered ascophyllan and crude extract significantly increased the serum levels of IFN-γ, but no significant increases in serum levels of IFN-γ in mice treated orally with ascophyllan and crude extract were observed (Figure 6C, F).

Discussion

Chemical analysis revealed that ascophyllan prepared in this study has a characteristic sugar composition ratio and level of sulfate group, as previously reported (13), while its

average molecular mass was estimated to be 98 kDa which was much smaller than 390 kDa reported previously (20). This may be due to the different lot of *A. nodosum* used as raw material. In the case of seaweed-derived polysaccharides, it is known that the molecular mass can vary depending on the parts of seaweed used, and on the harvest locations and seasons of seaweed (14, 20). The macrophage-stimulating activity of ascophyllan prepared in this study was confirmed in a *in vitro* system using the RAW264.7 mouse macrophage cell line, and even a higher activity was observed compared to previous reports. Crude extract also revealed macrophage-stimulating activity, but less effective than ascophyllan (data not shown). The lower activity of the crude extract might be due to the presence of inactive substances. To further determine the biological activities of ascophyllan, we evaluated the antitumor activity of ascophyllan and crude extract in sarcoma-180 solid tumor-bearing mice *via* two different routes of administration, continuous *i.p.* and oral administration.

Our previous studies found that ascophyllan exhibited antitumor activity in sarcoma-180 ascites tumor-bearing mice model at the dose of 50 mg/kg b.w./day by *i.p.* administration for six consecutive days (17). Injection *i.p.* of the same dose of ascophyllan for four consecutive days resulted in a significant increase in splenic NK cell activity (19). Based on previous studies, in the present study, 50 mg/kg b.w./day and 500 mg/kg b.w./day were chosen as effective *i.p.* and oral administration doses, respectively. The results obtained in our study show that oral administration of ascophyllan resulted in significant inhibition of the growth of sarcoma-180 solid tumor as judged from the reduction of both tumor volumes and tumor weights (Table II; Figure 2B). The antitumor effect of ascophyllan administered *via* the oral route was even greater than that observed for the *i.p.* route (Table II; Figure 2). Although the crude extract was less effective than ascophyllan, a statistically significant reduction of tumor volumes in sarcoma-180 tumor-bearing mice treated with crude extract *via* the oral route was observed (Table II; Figure 2B). Absorption of therapeutic drugs administered *i.p.* is faster and more efficient than that *via* the oral route. However, ascophyllan had a more potent antitumor activity through the oral than through the *i.p.* route. Similarly to our findings, polysaccharide alginates from brown seaweed *Sargassum vulgare* exhibited a more effective growth-inhibitory effect on sarcoma-180 solid tumors with oral administration than with *i.p.* administration (21). Therefore, it seems likely that seaweed-derived polysaccharides tend to exert more efficient antitumor effects with the oral route than by *i.p.* route as a common property. Furthermore, 30% and 40% of mice treated with ascophyllan and crude extract through the *i.p.* route died during the experiments, respectively, while no death of mice treated with oral administration were recorded (Table II). Hence, *i.p.*
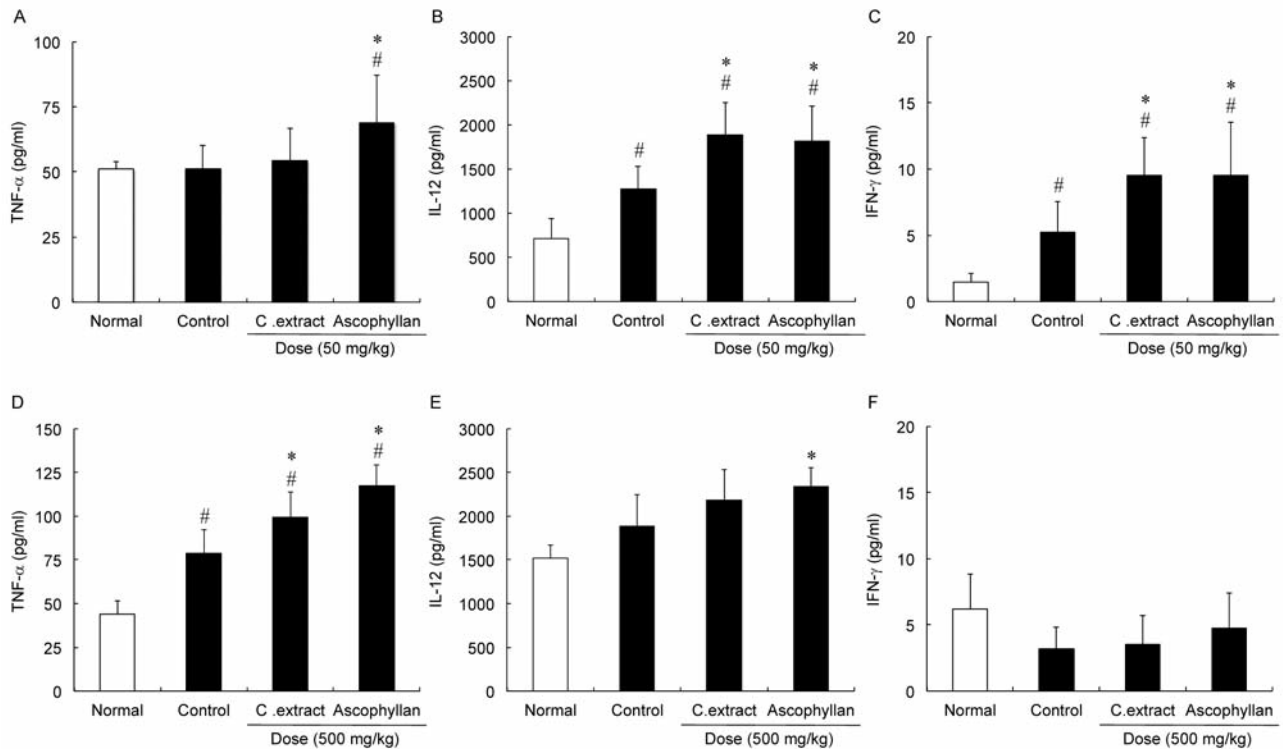
Figure 6. *Effects of ascophyllan and crude extract on the serum levels of tumor necrosis factor-α (TNF-α), interleukin-12 (IL-12), and interferon-γ (IFN-γ) in tumor-bearing mice. The serum levels of TNF-α, IL-12 and IFN-γ in normal mice, control tumor-bearing mice, and ascophyllan- or crude extract-treated tumor-bearing mice with continuous i.p. administration (A-C, respectively) or continuous oral administration (D-F, respectively). Data represent means±S.E. (n=10 for i.p. administration, n=8 for oral administration). Significant difference between *ascophyllan- or crude extract-treated tumor-bearing mice and control tumor-bearing mice (p<0.05), and #tumor-bearing mice and normal mice (p<0.05) were found.*

administered ascophyllan and crude extract may have negative side-effects on the health condition of the mice. These findings may support the idea that the oral route is an effective and safe route for ascophyllan in order to attain better antitumor effect.

Inoculated tumors cause a leukemoid reaction in test animals characterized by granulocytosis and splenomegaly (23). In fact, the weights of spleens from sarcoma-180 tumor-bearing mice were significantly increased compared to those of normal mice (Figure 4). Treatment with ascophyllan *i.p.* induced further increase in spleen weights (Figure 4A), while treatment by the oral route resulted in a rather slight reduction (Figure 4B). These results suggest that the influence of ascophyllan on the host immune system can differ significantly depending on the administration route. Similarly to our results, it has been reported that oral administration of alginates from brown seaweed *S. vulgare* to sarcoma-180 tumor-bearing mice resulted in the reduction of spleen weights, whereas *i.p.* injection further increased spleen weights as compared to untreated tumor-bearing mice (21).

Host immune responses to tumors are mainly mediated by various types of effector cells, such as tumor-specific cytotoxic T-cells, non-specific tumoricidal macrophages, and NK cells. In particular, NK cells play a critical role in host defense against tumors (24). Administration of ascophyllan *i.p.* significantly enhanced the splenic NK cell activity in tumor-bearing mice, while only a slight effect was found in orally ascophyllan-treated groups (Figure 5). Crude extract had no significant effect on splenic NK cell activity (Figure 5). Although ascophyllan administered with *i.p.* route may influence the spleen more effectively than by the oral route, a better antitumor effect of ascophyllan with oral administration suggests that factors other than increased splenic NK cell activity may be mainly responsible for the antitumor activity of orally-administered ascophyllan.

In general, oral ingestion is the most common, most convenient, and most economical method for drug administration, and the availability of chemotherapeutic drugs applicable for oral route is considered to be a step forward in cancer treatment (21, 25). Since ascophyllan showed even better antitumor effect with the oral rather than the *i.p.* route, this polysaccharide may meet this criterion as a therapeutic agent.

In addition to the splenic NK cell activity, continuous *i.p.* administration of ascophyllan resulted in significant increase

in the serum levels of TNF-α, IL-12, and IFN-γ in treated mice. Administration of crude extract *i.p.* also induced an increase in serum levels of IL-12 and IFN-γ, but that for the TNF-α level was not significant (Figure 6A, B and C). Hence, our results suggest that *i.p.*-administered ascophyllan can exhibit antitumor activity through the activation of both cellular and humoral host immune systems. Synergistic action of TNF-α and IL-12 leads to augmentation of the antitumor effect even though either of these cytokines alone was ineffective (26, 27). Since orally-administered ascophyllan significantly increased the serum levels of TNF-α and IL-12 with even higher levels than those induced by the *i.p.* route (Figure 6A, B, D and E), it is suggested that the antitumor activity of orally administered ascophyllan is partly attributed to the synergistic action of increased TNF-α and IL-12. Since the profiles of increased cytokines in the serum induced by ascophyllan differed depending on the administrative route, especially for IFN-γ, the effects of ascophyllan on the host immune system may be somewhat different depending on the administrative route. Furthermore, higher antitumor and immunostimulating effects of ascophyllan than the crude extract suggest that ascophyllan may be a main active polysaccharide in the crude extract responsible for the antitumor activity.

Recently, it has been demonstrated that a small amount of fucoidan is systemically-ingested from oral dosing by using antibody-based methods (28). Interestingly, Tokita *et al.* demonstrated that fucoidan was unchanged in the serum or plasma of human beings through recording molecular weight profiles of the fucoidan after oral administration (29). These findings might support the notion that a part of orally-administered ascophyllan molecules can be ingested and reach the host immune system, and stimulate immune-competent cells to allow certain cells to release cytokines and other agents which might be involved in the antitumor activity. It has recently been reported that oral administration of polysaccharides extract of brown alga *Kjellmaniella crassifolia* significantly enhanced the intestinal immune response through stimulating the T-lymphocytes of Peyer's patch cells to release IL-10 and IFN-γ, but no activated phenomenon was observed in splenic immune-competent cells (30). It has also been reported that orally-administered β-glucans from an oriental mushroom (*Grifola frondosa*) exhibited antitumor activity through stimulating immune cells such as macrophages and dendritic cells present in the Peyer's patch (31). These findings suggest that even orally-administered polysaccharides with a high molecular weight can reach host immune systems, and exert antitumor effects through adjuvant activity. The greater antitumor effects of orally-administered ascophyllan rather than those by the *i.p.* route may support this idea. Further studies are required to clarify the detailed antitumor mechanisms of orally-administered ascophyllan, including the possible involvement of intestinal immune system.

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