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Oversulfation of fucoidan enhances its anti-angiogenic and antitumor activities

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Abstract

Fucoidan, a sulfated polysaccharide extracted from brown seaweed, has anticoagulant and antithrombotic activities. Unlike heparin, it shows an inhibitory action on the progression and metastasis of malignant tumors, although the precise mechanisms have not been elucidated. We have demonstrated previously that fucoidan can inhibit tube formation following migration of human umbilical vein endothelial cells (HUVEC) and that its chemical oversulfation enhances the inhibitory potency. In this study, we tested the hypothesis that fucoidan may suppress tumor growth by inhibiting tumor-induced angiogenesis. Both natural and oversulfated fucoidans (NF and OSF) significantly suppressed the mitogenic and chemotactic actions of vascular endothelial growth factor 165 ($VEGF_{165}$) on HUVEC by preventing the binding of $VEGF_{165}$ to its cell surface receptor. The suppressive effect of OSF was more potent than that of NF, suggesting an important role for the numbers of sulfate groups in the fucoidan molecule. Consistent with its inhibitory actions on $VEGF_{165}$, OSF clearly suppressed the neovascularization induced by Sarcoma 180 cells that had been implanted in mice. The inhibitory action of fucoidan was also observed in the growth of Lewis lung carcinoma and B16 melanoma in mice. These results indicate that the antitumor action of fucoidan is due, at least in part, to its anti-angiogenic potency and that increasing the number of sulfate groups in the fucoidan molecule contributes to the effectiveness of its anti-angiogenic and antitumor activities.

Keywords: Fucoidan; Angiogenesis; Vascular endothelial growth factor; Endothelial cell (human umbilical vein)

1. Introduction

Angiogenesis, new blood vessel formation, is an indispensable biological event for a variety of physiological or pathological processes [1,2]. Highly regulated and transient angiogenesis is necessary for embryonic development, wound healing, and corpus luteum formation. However, uncontrolled and persistent angiogenesis occurs in several pathological states such as diabetic retinopathy, rheumatoid arthritis, and tumor progression. Tumor growth requires angiogenesis to supply nutrients and oxygen [3]. In addition, it utilizes the newly formed blood vessels as conduits to disseminate invasive tumor cells. Because the growth and metastasis of malignant tumors are dependent upon angiogenesis, a novel anticancer treatment has been developed in which tumors are regressed by prolonged inhibition of angiogenesis. Thus, a variety of anti-angiogenic agents are currently undergoing clinical trails for dormancy therapy of tumors [4].

Tumor-induced angiogenesis is regulated by cell-produced factors that have mitogenetic and chemotactic effects on vascular endothelial cells. Several endothelial growth factors such as fibroblast growth factors, transforming growth factor-β, platelet-derived growth factor, and VEGF have been identified as angiogenic factors [5–7]. In particular, the expression of VEGF in tumor cells is considered to play a major role in tumor-induced angiogenesis [8,9]. As a high expression of VEGF and its receptors in the tumor is closely correlated to its vascularity, progression, and metastasis, targeting of VEGF and/or VEGF receptors is thought to be an effective strategy of anti-angiogenic therapy [10,11]. Several sulfated polysaccharides have been shown to modulate the proliferation and migration of vascular endothelial

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Abbreviations: NF, natural fucoidan; OSF, oversulfated fucoidan; VEGF, vascular endothelial growth factor; FGF-2, fibroblast growth factor-2; HUVEC, human umbilical vein endothelial cells; VEGFR-2, vascular endothelial growth factor receptor-2; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PMSF, phenylmethylsulfonyl fluoride.

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cells by altering the binding of endothelial growth factors to their cell surface receptors [12–14].

Fucoidan, which is a sulfated polysaccharide present in brown marine algae, has been reported to exhibit antitumor and antimetastatic activities in xenograft mouse models [15,16]. However, the molecular mechanism by which fucoidan suppresses tumor growth and metastasis has not been clarified. We previously showed that NF efficiently suppresses FGF-2-induced migration and tube formation of HUVEC, and that the potency of the agent can be increased by chemical sulfation [17]. We have also shown that OSF inhibits the migration and tube forming ability of HUVEC on Matrigel made from murine Engelbreth-Holm-Swarm sarcoma [18]. From these data, we hypothesized that fucoidan might exert its antitumor activity through the inhibition of tumor-induced angiogenesis.

In the present study, we provide evidence that both NF and OSF suppress the growth of tumor cells implanted in mice by preventing tumor-induced angiogenesis. We also investigated the possible effects of NF and OSF on the activities of the most specific angiogenic growth factor, VEGF. Our data demonstrated that both fucoidans prevent the VEGF-induced phosphorylation of VEGFR-2 by their binding to VEGF itself, and that the degree of sulfation in the fucoidan molecule plays an important role in interfering with VEGF binding to VEGFR-2.

2. Materials and methods

2.1. Materials

The following materials were commercially obtained: *Fucus vesiculosus* fucoidan from the Sigma Chemical Co.; recombinant human $VEGF_{165}$ from Oncogene Research Products; DMEM and FBS from GIBCO BRL, Life Technologies, Inc.; and porcine Type IV collagen from the Nitta Gelatin Co.

2.2. Purification and oversulfation of fucoidan

NF (200 mg) was purified on a Sephadex G-100 column (2.5 × 36 cm) equilibrated with 0.5 M NaCl. The content of fucoidan in each fraction was determined by the anthrone-H_2SO_4 method. Fractions corresponding to a 100–130 kDa molecular mass region were collected and dialyzed against water at 25° for 16 hr. The dialysate (purified fucoidan) was lyophilized and stored at 4° until used. OSF with a molecular mass of 100–130 kDa was prepared as described previously [9]. Briefly, the purified NF was sulfated further in a mixture of dimethylformamide and sulfur trioxide–trimethylamine complex at 50° for 24 hr. The product was recovered by ethanol precipitation, followed by purification on the Sephadex G-100 column. The yield of OSF was 107 mg; the sulfate content was estimated to be 56.7% (the NF was estimated to be 32.6%). Electrophoretic analysis revealed that both NF and OSF were nearly homogeneous, as shown previously [19].

2.3. Cell cultures

Cyro HUVEC and the culture medium EGM-2 were purchased from Sanko Junyaku. EGM-2 is composed of modified MCDB 131 medium and supplements (FBS, epidermal growth factor, VEGF, FGF-2, insulin-like growth factor, heparin, ascorbic acid, hydrocortisone, amphotericin B, and gentamicin). HUVEC were maintained in EGM-2 at 37° in a humidified 5% CO_2 atmosphere. Lewis lung carcinoma cells were supplied by The Cell Resource Center for Biomedical Research, Tohoku University. Two murine tumor cell lines (B16 melanoma and Sarcoma 180) were purchased from Dai Nippon Seiyaku. These tumor cells were maintained in DMEM supplemented with 10% FBS at 37° in a humidified 5% CO_2 atmosphere.

2.4. Cell proliferation assay

HUVEC (2×10^3) were seeded in 24-well plates and allowed to attach to the well bottom for 24 hr in low serum EGM-2 medium (0.1% FBS). The attached cells were treated with NF or OSF, followed by the addition of $VEGF_{165}$ at 10 ng/mL. After 48 hr of culturing, [^{3}H]thymidine (10 μL, 25 μCi) was added to each well, and incubation followed at 37° for 4 hr. After being washed three times with PBS, the cells were lysed with 500 μL PBS containing 5% Triton X-100 and 1% SDS. The cell lysates were transferred into scintillation vials containing 10 mL of aqueous counting scintillant (Amercharm Pharmacia Biotech). Radioactivity was measured using a liquid scintillation counter.

2.5. Cell migration assay

HUVEC (1×10^3) were seeded into collagen IV-coated transwell chambers (Falcon), and the chambers were inserted into 24-well plates containing 1 mL of EGM-2 medium. After incubation for 1 hr, $VEGF_{165}$ (10 ng/mL) alone or with NF or OSF was added to each well. Following incubation for 8 hr, the cells that remained on the upper side of the transwell membrane were removed with cotton swabs. The cells were fixed and stained with Diffi-Quick (American Scientific Products), and counted in 3 fields under a ×100 high power field (HPF).

2.6. Detection of phosphorylated VEGFR-2

Confluent cultures of HUVEC in 175-cm^2 culture flasks were incubated for 3 hr in 20 mL of low serum EGM-2 medium. After removal of the medium, the cells were incubated for 10 min in medium containing $VEGF_{165}$ (10 ng/mL) with NF or OSF (10 μg/mL each).

The incubation was terminated by aspiration of the medium. After being washed twice with cold PBS containing 200 μM $Na_2V_2O_5$, the cells were lysed in ice for 10 min with 100 μL of 50 mM Tris–HCl buffer (pH 7.8)/150 mM NaCl/5 mM EDTA/2 mM PMSF/0.5% Nonidet P-40/10 μg/mL of leupeptin/10 μg/mL of antipain. After removal of insoluble materials by centrifugation at 12,000 *g* for 15 min at 4°, the resulting supernatants were assayed to detect phosphorylated VEGFR-2. Protein concentration was determined by using a BCA protein assay kit (Sigma). The supernatant proteins (30 μg) were separated on SDS–polyacrylamide gels, and transferred to polyvinylidene difluoride membranes. The membranes were reacted with antibodies against phospho-VEGFR-2 (Oncogene Research Products). The immunocomplexes were reacted further with peroxidase-conjugated secondary antibodies and made visible with 4-chloro-1-naphthol as the peroxidase substrate.

2.7. [^{125}I]VEGF$_{165}$ binding assay

HUVEC were cultured to subconfluence in 24-well culture plates. The binding experiment was performed as follows. [^{125}I]VEGF$_{165}$ (100 pmol, Amercharm Pharmacia Biotech) and NF or OSF (10 μg/mL each) were mixed with 1 mL of EGM-2 medium containing 20 mM HEPES/0.2% gelatin and allowed to stand at 25° for 2 hr. The mixture was added to each well, and incubation followed at 4° for 2 hr. After removal of unbound radioligands, the cell layers were washed three times with ice-cold PBS. The washed cells were lysed with 500 μL of PBS containing 5% Triton X-100 and 1% SDS. The amount of [^{125}I]VEGF$_{165}$ bound to HUVEC was measured, using a gamma counter.

2.8. Fucoidan binding to immobilized VEGF$_{165}$

The binding of NF or OSF to VEGF$_{165}$ was quantified by using the BIAcore™ 1000 system (Pharmacia Biosensor AB). The CM5 sensor chip in the BIAcore system was first activated by injection of 50 μL of a mixture of 0.1 M *N*-hydroxysuccinimide/0.4 M *N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide according to the protocol of the manufacturer. VEGF$_{165}$ (10 μg) was dissolved in 100 μL of 10 mM glycine–HCl buffer (pH 4.0)/15 mM NaCl, and the solution was injected over the activated chip surface at a flow rate of 5 μL/min at 25°. The remaining active sites were blocked by the injection of 50 μL of 1.0 M ethanolamine HCl (pH 8.5) at a flow rate of 10 μL/min. Various concentrations of NF or OSF dissolved in PBS were injected over the VEGF-immobilized sensor chip at a flow rate of 10 μL/min. The binding kinetics were analyzed using BIA evaluation software (ver. 3.0).

2.9. Animals and treatments

Male ICR mice (5 weeks old) were purchased from Charles River Japan Inc. They were housed in a light-controlled room (light on from 7:00 a.m. to 7:00 p.m.) at a room temperature of 24 ± 1° and a humidity of 60 ± 10% with food and water *ad lib*. The animals were treated in accordance with Law (No. 105) and Notification (No. 6) of the Japanese Government.

2.10. In vivo angiogenesis

The tumor-induced neovascularization was assessed by the dorsal air-sac method [9]. Briefly, cultured Sarcoma 180 cells (1×10^7) were packed into a membrane chamber, and the chamber was implanted into the dorsal air sac of mice (day 0). The mice were injected with NF or OSF (daily dose, 5 mg/kg each; i.v.) from day 1 to 6. This optimal dosage was determined after preliminary experiments (data not shown). Control mice were injected with saline alone. The mice were deeply anesthetized with ether and killed on day 7. The newly formed blood vessels, in the subcutaneous regions adjacent to the implanted chamber, were photographed under a dissecting microscope.

2.11. In vivo tumor growth

Lewis lung carcinoma or B16 melanoma cells (1.5×10^6) were inoculated into the right hind footpads of mice. The mice were injected i.v. with a single daily dose of NF or OSF (5 mg/kg each) starting 3–5 days after inoculation with the tumor cells. Control mice were injected with saline alone. Tumor volumes and animal body weights were measured throughout the duration of the experiment. The tumor volume was estimated according to the following formula: tumor volume $(cm^3) = 4\pi(xyz)/3$, where x, y, and z are the three perpendicular diameters of the tumor.

3. Results and discussion

3.1. Effects of NF and OSF on the VEGF$_{165}$-induced proliferation and migration of HUVEC

VEGF is a potent selective mitogenic cytokine for vascular endothelial cells [8,20,21]. Five isoforms of human VEGF mRNA, which encode VEGF proteins of 121, 145, 165, 189, and 206 amino acids are produced from a single gene, as the result of alternative splicing [22]. The best characterized VEGF is the 165-amino acid form, VEGF$_{165}$, which induces angiogenesis and blood vessel permeability *in vivo* and displays a mitogenic activity restricted to vascular endothelial cells [23]. As shown in Fig. 1A, NF and OSF significantly inhibited the VEGF$_{165}$-induced proliferation of HUVEC in a concentration-dependent manner. NF significantly inhibited the HUEVC proliferation at a concentration of 100 μg/mL ($P < 0.01$), while OSF caused a significant inhibition at 10 μg/mL ($P < 0.05$). Fig. 1B shows the effects of both fucoidans on VEGF$_{165}$-induced migration of HUVEC. Significant

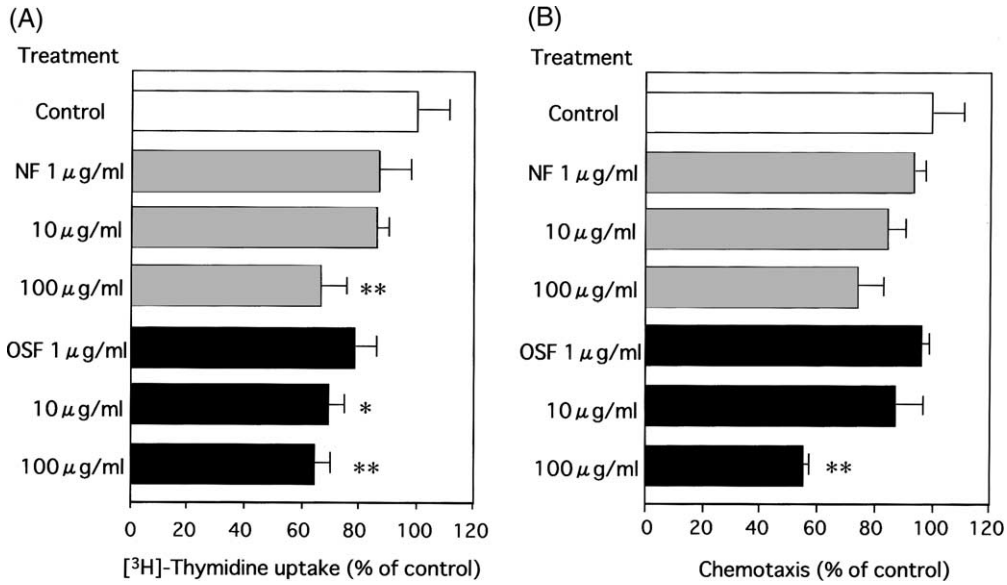

Fig. 1. Effects of NF and OSF on the $VEGF_{165}$-induced proliferation (A) and migration (B) of HUVEC. Each bar represents the mean ± SEM of 4–6 independent experiments. Control (absolute) values: panel A, 869 ± 105 cpm; panel B, 28.9 ± 3.2 cells/HPF ×100. Key: (*) $P < 0.05$ and (**) $P < 0.01$, compared with the control (by Dunnett's test).

inhibition was observed only when HUVEC were treated with $VEGF_{165}$ in the presence of 100 μg/mL of OSF ($P < 0.01$). These results indicate that, in the concentration range examined, both NF and OSF at least have the ability to inhibit $VEGF_{165}$-induced proliferation and migration of HUVEC, but inhibit the proliferation more effectively than the migration. The data also suggest that the inhibitory potencies of fucoidans depend on the degree of sulfation.

3.2. Effects of NF and OSF on the phosphorylation of VEGFR-2

To clarify the underlying mechanism by which fucoidans inhibit the proliferation and migration of HUVEC, we investigated the effects of NF and OSF on $VEGF_{165}$-mediated signal transduction in HUVEC. $VEGF_{165}$ is thought to exert its angiogenic effect by binding to VEGFR-2 (also known as KDR), which leads to autophosphorylation and consequently a series of specific downstream signaling events [24]. As shown in the upper panel in Fig. 2, treatment of HUVEC with $VEGF_{165}$ for 10 min resulted in extensive phosphorylation of VEGFR-2, with a molecular mass of approximately 210 kDa, when compared with the control (lane 1 versus lane 2). The degree of phosphorylation of VEGFR-2 apparently decreased in the presence of 10 μg/mL of NF and OSF (lanes 3 and 4, respectively). The effect of OSF was more potent than that of NF. The lower panel in Fig. 2 also shows that there was no difference in the amount of total (phosphorylated and non-phosphorylated) VEGFR-2 protein in each lane, indicating that the decreases in phosphorylated VEGFR-2 seen in lanes 3 and 4 were due to the effects of NF and OSF, respectively. These results support the above findings that OSF inhibits the $VEGF_{165}$-induced proliferation and migration of HUVEC more effectively than does NF, and suggest that these inhibitory effects are caused by the prevention of VEGF-mediated signal transduction.

3.3. Effects of NF and OSF on $VEGF_{165}$ binding to HUVEC

It has been reported that heparin and fucoidan modulate the proliferation and migration of HUVEC mainly by altering the binding of FGF-1 and FGF-2 to their cell surface receptors [17,25]. $VEGF_{165}$ also binds to heparin through a site separated from its receptor-binding domain [26], and it has been suggested that the formation of this complex results in the modulation of $VEGF_{165}$ binding to VEGFR-2. Therefore, we assumed that both fucoidans might have the ability to modulate $VEGF_{165}$ binding to vascular endothelial cells after forming the complex. As expected, both fucoidans at 10 μg/mL prevented the bind-

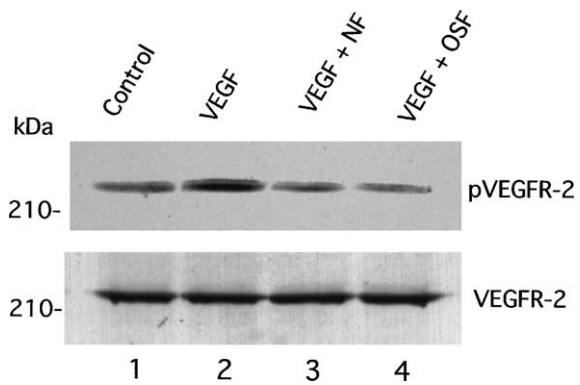

Fig. 2. Effects of NF and OSF on the $VEGF_{165}$-induced autophosphorylation of VEGFR-2.

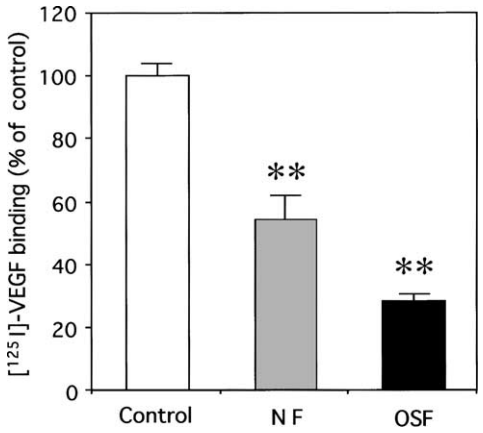

Fig. 3. Effects of NF and OSF on the binding of $VEGF_{165}$ to HUVEC. Each bar represents the mean ± SEM of 3 independent experiments. Control (absolute) value: 820 ± 32 cpm. Key: (**) $P < 0.01$, compared with the control (by Dunnett's test).

ing of [^{125}I]VEGF to HUVEC, and the inhibitory action of OSF was more potent than that of NF (Fig. 3). This finding suggests that both fucoidans decrease the $VEGF_{165}$-induced phosphorylation of VEGFR-2 by lowering the amount of receptor-bound $VEGF_{165}$. The prevention of $VEGF_{165}$ binding to VEGFR-2 may be caused by the interaction of fucoidans with $VEGF_{165}$.

3.4. Analysis of the fucoidan binding to $VEGF_{165}$

We next used surface plasmon resonance analyses (BIAcore™ 1000 system) to test the abilities of both fucoidans to bind directly to $VEGF_{165}$. Fig. 4 shows that both NF and OSF bound to the $VEGF_{165}$-conjugated sensor chip with fast kinetics. The NF association rate constant was 3.58×10^3 M^{-1} sec^{-1}, and the dissociation rate constant was approximately 1.32×10^{-3} sec^{-1}. Steady-state binding analysis showed that the affinity (K_d) of NF for immobilized $VEGF_{165}$ was 368 nM. The association and dissociation rate constants of OSF were 4.85×10^3 M^{-1} sec^{-1} and 2.53×10^{-4} sec^{-1}, respectively. Thus, the interaction of OSF with $VEGF_{165}$ occurs with a high affinity (K_d = 52 nM) and results in the formation of highly stable complexes. These data strongly suggest that both fucoidans can directly bind to $VEGF_{165}$, thereby preventing $VEGF_{165}$ binding to the receptor. The results of the kinetic analysis also revealed that the binding affinity (K_d) of OSF to $VEGF_{165}$ was approximately 7-fold higher than that of NF. The difference in the affinities of the two fucoidans may affect the efficiency of fucoidan–$VEGF_{165}$ complex formation and simultaneously reflect the inhibitory potencies of fucoidans. Fucoidan is a polymer of l-fucose, having a sulfate group on either the C3 or C4 position of the fucose unit. OSF has sulfated groups on both positions [19,27]. Therefore, the sulfate groups introduced onto OSF may produce a particular new spatial orientation of the negative charges, which may play a key role in determining the binding potency to $VEGF_{165}$.

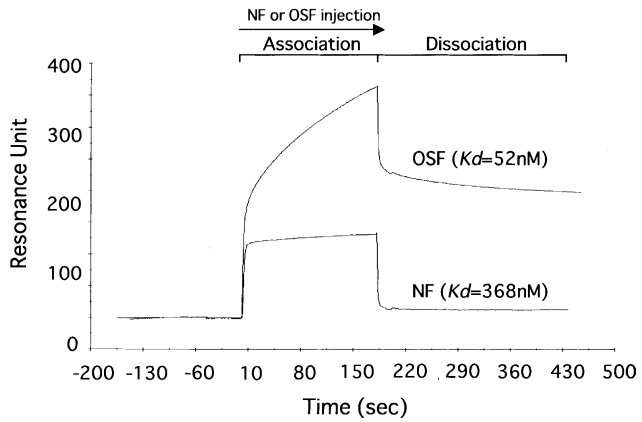

Fig. 4. Kinetic analysis of the interaction of $VEGF_{165}$ with NF or OSF. Shown are representative sensorgrams of injecting 10 μg/mL of NF or OSF into the $VEGF_{165}$-conjugated sensor chip.

3.5. Effects of NF and OSF on angiogenesis and tumor growth

We next tested whether NF and OSF could exhibit their anti-angiogenic actions *in vivo* (Fig. 5A). NF or OSF (5 mg/kg each, i.v.) was administered to mice in which a membrane chamber including Sarcoma 180 cells had been implanted. The control mice received saline alone. Seven days after implanting the tumor cells, neovascularization from surrounding blood vessels was observed in the control mice in the region adjacent to the implanted chamber. On the other hand, neovascularization in mice given NF or OSF was clearly suppressed. Photographs show that the blood vessels in the OSF-treated mice were virtually the same as those of the PBS control mice (no tumor cells).

The effects of NF and OSF on the growth of Lewis lung carcinoma or B16 melanoma cells inoculated into the right hind footpads of mice are shown in Fig. 5B. The growth of the two types of murine tumor cells was clearly suppressed by the repetitive administration of each fucoidan (5 mg/kg, i.v.). Also, the effect of OSF was more potent than that of NF. During these experiments, significant adverse effects such as weight loss and platelet aggregation were not observed in the mice treated with NF and OSF. These results suggest that the antitumor action of fucoidan is due, at least in part, to its anti-angiogenic potency and that the degree of sulfation in the fucoidan molecule plays an important role in the anti-angiogenic and antitumor activities of the molecule.

Because the growth of solid tumors requires an adequate blood supply, agents that inhibit angiogenesis induce the suppression of tumor growth. To determine whether the observed inhibition of tumor growth by fucoidans is due to their anti-angiogenic activities or to a direct effect on the tumor cells, we used a tetrazolium bromide (MTT) assay to examine the effects of NF and OSF on the viability of three types of cultured murine tumor cells (Sarcoma 180, Lewis

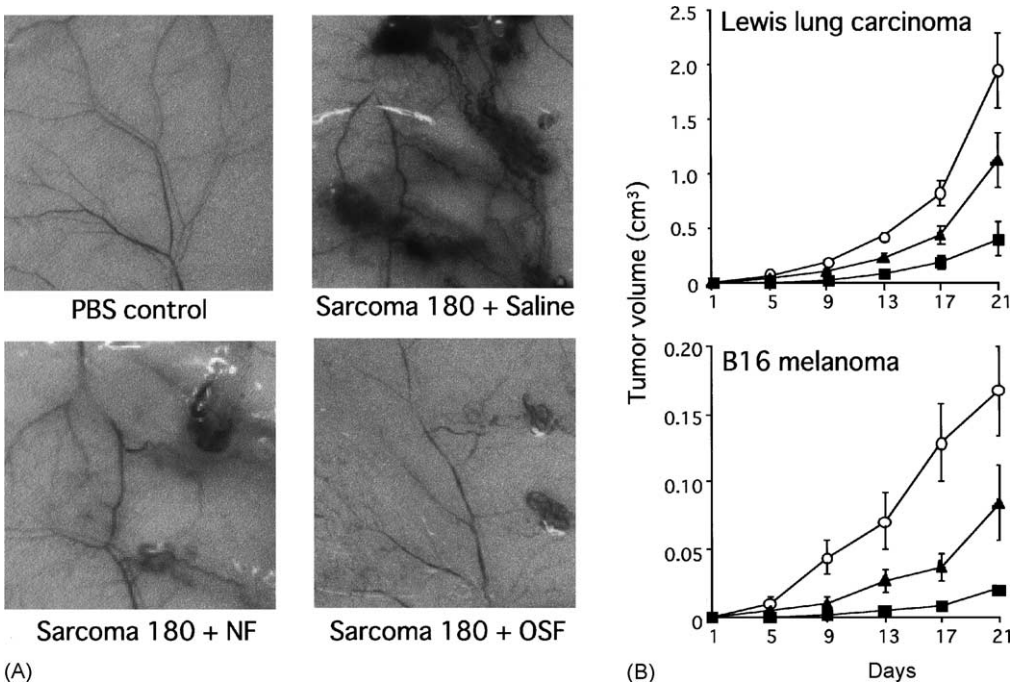

Fig. 5. Effects of NF and OSF on angiogenesis and tumor growth in mice. (A) Photographs showing the neovascularization induced by Sarcoma 180 cells packed in membrane chambers in mice. The control chamber contained PBS instead of Sarcoma 180 cells. (B) Tumor-bearing mice were treated for 21 days with a single daily dose of NF (▲), OSF (■) (5 mg/kg each, i.v.), or saline (○, control). Each point is the mean ± SEM of 7–11 mice.

lung carcinoma, and B16 melanoma). However, even at a concentration of 100 μg/mL, no cytotoxic effect from either fucoidan was observed (data not shown). Therefore, the two fucoidans suppress tumor growth through the prevention of tumor-induced angiogenesis rather than a direct cytotoxic effect on tumor cells.

Taken together, these results indicate that NF and its oversulfated derivative, OSF, are potent anti-angiogenic agents, when considering their inhibitory effects on $VEGF_{165}$-induced proliferation and migration of vascular endothelial cells. The anti-angiogenic activity of fucoidan is likely due to the inhibition of $VEGF_{165}$ binding to its cell surface receptors and, consequently, of the downstream signal transduction. Our present data show that by increasing the number of sulfate groups in the fucoidan molecule its anti-angiogenic and antitumor activities can be potentiated. The role of angiogenesis in tumor progression and invasiveness is well-recognized, and anti-angiogenesis is becoming a new therapeutic approach for the treatment of cancers. Therefore, further studies elucidating the precise mechanism of the anti-angiogenic action of fucoidan may provide a basis for the development of more effective analogs to induce, selectively, the regression of malignant tumors.

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