Inhibitory Effect of Oversulfated Fucoidan on Tube Formation by Human Vascular Endothelial Cells

Shinji SOEDA, Yoshiaki SHIBATA, and Hiroshi SHIMENO*

Department of Biochemistry, Faculty of Pharmaceutical Sciences, Fukuoka University, 8–19–1 Nanakuma, Jonan-ku, Fukuoka 814–80, Japan. Received June 16, 1997; accepted August 8, 1997

Fucoidan is a sulfated poly(L-fucopyranose) present in brown marine algae. In this study, we examined the effect of native and chemically oversulfated fucoidans (NF and OSF) on the tube structure formation by human umbilical vein endothelial cells (HUVEC) on the basement membrane preparation, Matrigel. Unlike NF, OSF significantly decreased the tube formation: maximal inhibition (50% of control) was obtained with 25 $\mu\text{g/ml}$. The OSF effect was mediated, at least in part, through the inhibition of HUVEC migration, as determined by the ability to block chemotaxis in a Transwell chamber assay. Quantitative immunoreactive assays for tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) in the culture media indicated that OSF (25 $\mu\text{g/ml}$) increased the accumulation of PAI-1 antigen, but not of t-PA antigen, 2.7-fold compared with control. The release of both antigens by HUVEC was slightly affected by the addition of NF. Determination of the media levels of type IV collagenase activity and tissue inhibitor of metalloproteinase-1 (TIMP-1) antigen showed that OSF (25 $\mu\text{g/ml}$) decreased the collagenolytic activity by 50% compared to the control, without alteration of the TIMP antigen level. However, the collagenase inhibition by OSF was not observed in an assay system using purified enzyme. NF had no effect on collagenase activity or TIMP-1 antigen levels. These results indicate that the introduction of sulfate groups into NF enables it to effectively inhibit the formation of capillary-like structures by HUVEC on Matrigel by reducing the basement membrane destruction and cell migration. It is involved as at least one of the mechanisms by which the OSF-induced increase in HUVEC PAI-1 decreases plasmin formation and suppresses the following pro-collagenase activation.

Key words angiogenesis; fucoidan; plasminogen activator inhibitor-1; collagenase; endothelial cell (human umbilical vein)

Angiogenesis is a sequential multi-stage process involving (1) basement membrane degradation by proteases, (2) endothelial cell migration, (3) proliferation, and (4) maturation into capillary vessels.¹⁾ When it occurs, during wound healing or in response to ovulation, it is tightly controlled and strictly delimited. By contrast, many pathological conditions, such as cancer, atherosclerosis and diabetic retinopathy, are characterized by persistent and unregulated angiogenesis. Control of the neovascularization could result in new therapeutic approaches to these disorders. Several anti-angiogenic agents are currently undergoing clinical trials for the treatment of cancer.²⁾ However, better understanding of the angiogenic process and the search for more ideal therapeutic agents continues.

Fucoidan (NF) is a sulfated poly(L-fucopyranose) present in brown marine algae and has been previously reported to have anti-metastatic activity.³⁾ Our recent study has shown that the ability of NF to inhibit 3LL cell invasion through the reconstituted basement membrane (Matrigel) is improved by its further sulfation, and that oversulfated fucoidan (OSF) effectively decreases the physical interaction between 3LL cells and the laminin component in Matrigel, followed by a suppression of the laminin-induced increase in urokinase-type plasminogen activator release.⁴⁾ These findings suggest that OSF acts as an inhibitor of cell migration that results from matrix proteolysis. In this paper, we describe the suppressive effect of OSF on the tube formation of cultured human umbilical vein endothelial cells (HUVEC) on Matrigel. The results indicate that OSF suppresses HUVEC angiogenesis mainly by increasing the release of plasminogen activator inhibitor-1 (PAI-1), which plays a key role in

regulating matrix remodeling by proteases.

MATERIALS AND METHODS

Materials The following materials were commercially obtained: *Fucus vesiculosus* fucoidan from Sigma Chemical Co., St. Louis, MO, U.S.A.; Matrigel from Collaborative Research, Inc., Bedford, MA, U.S.A.; Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) from GIBCO BRL, Life Technologies, Inc., Rockville, MD, U.S.A.; *Clostridium histolyticum* type IV collagenase from Wako Pure Chemical Co., Osaka, Japan; mouse anti-human tissue inhibitor of metalloproteinase-1 (anti-TIMP-1) from Fuji Chemical Industries, Ltd., Takaoka, Japan; goat anti-mouse Ig G peroxidase conjugate from EY Laboratories, Inc., San Mateo, CA, U.S.A.

HUVEC Culture HUVEC (passage 2) and the culture medium EGM-UVTM were purchased from Sanko Junyaku Co., Ltd., Tokyo, Japan. The cells (6×10^5 cells) were plated into a 175-cm² tissue culture flask (Falcon) and grown to near confluency in EGM-UVTM (modified MCDB 131 medium that was supplemented with 2% fetal calf serum, 10 ng/ml epidermal growth factor, 1 $\mu\text{g/ml}$ hydrocortisone, 50 $\mu\text{g/ml}$ gentamycin, 0.25 $\mu\text{g/ml}$ amphotericin B, 10 $\mu\text{g/ml}$ heparin and 10 $\mu\text{g/ml}$ bovine brain extract) at 37 °C in a humidified 5% CO₂ atmosphere. The medium was renewed every 2 d. Subcultures were obtained by treating the HUVEC cultures with 0.05% trypsin/0.53 mM EDTA (GIBCO) at 37 °C for 5 min. All experiments were performed with HUVEC in the 4th to 5th passages.

Assay of Tube Formation Matrigel (350 μl) was placed into each well of a 24-well culture plate at 4 °C and was

* To whom correspondence should be addressed.

allowed to polymerize by incubation at 37 °C for 30 min. HUVEC (3.5×10^4 cells) were seeded on the Matrigel in 700 μ l DMEM supplemented with 20% FBS, and incubation followed at 37 °C for 24 h in a humidified 5% CO₂ atmosphere. Four different phase-contrast microscopic fields ($\times 100$) per well were photographed, and the micrographic pictures were put on a computer. The total length of tube structures in each photograph was measured using Adobe Photoshop™ software. The total capillary length per well was given as the average value of the data from four fields.

Assay of HUVEC Migration Polycarbonate filter Transwell inserts (24 well size) with 8- μ m pores (Costar, Cambridge, MA, U.S.A.) were used for this invasion assay. Matrigel (50 μ l from a 250 μ g/ml dilution in cold PBS) was applied to the upper surface of each filter and dried at 25 °C. The diluted Matrigel (600 μ l) was placed in the lower chamber. HUVEC (10^4 cells/100 μ l DMEM containing 10% FBS) was added to the upper chamber and incubated at 37 °C for 2 d. Cells that remained on the upper side of the filter were removed with cotton swabs. Filters were then fixed and stained with Diffi-Quik (American Scientific Products). The stained cells were counted in 5 fields under a $\times 100$ high power field (HPF).

Assay of HUVEC Attachment Matrigel was diluted with cold PBS at a concentration of 50 μ g/ml, and 200 μ l of the solution was incubated in each well of a 96-well plate at 4 °C overnight. The wells were thereafter treated with 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at 25 °C for 2 h, and washed 3 times with 200 μ l PBS. To each well was added 50 μ l NF (or OSF) solution and 50 μ l HUVEC suspension (1×10^5 cells/ml in DMEM containing 10% FBS). The cells were allowed to adhere at 37 °C for 60 min. Unbound cells were removed by washing the wells with 200 μ l PBS. The adherent cells were fixed with 2.5% glutaraldehyde for 10 min and stained with Giemsa's stain (Merck). The stained cells were microscopically counted (5 fields under a $\times 100$ HPF).

Purification and Oversulfation of Fucoidan NF (200 mg) was purified on a Sephadex G-100 column (2.5 $\times$ 36 cm) equilibrated with 0.5 M NaCl. OSF with a molecular mass of 100–130 kDa was prepared as described previously.⁵⁾ Briefly, the purified NF was further sulfated in a mixture of dimethylformamide and sulfur trioxide-trimethylamine complex at 50 °C for 24 h. The product was recovered by ethanol precipitation, followed by purification on the Sephadex G-100 column. The yield of OSF was 144 mg; the sulfate content was estimated to be 52.4%.

Assay of t-PA and PAI-1 Antigens Antigen levels of t-PA and PAI-1 in the conditioned media were determined with ELISA kits, TintElize t-PA and TintElize PAI-1 (Biopool AB, Umeå, Sweden), respectively, according to the manufacturer's instructions.

Assay of TIMP-1 Antigen The media containing TIMP-1 (10 μ l each) were subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under unreduced conditions.⁶⁾ The separated proteins were transferred to a 0.22- μ m nitrocellulose membrane (Millipore). The following immunoblotting

procedures were as described previously,⁷⁾ except that mouse anti-hTIMP-1 and goat anti-mouse Ig G peroxidase conjugate were used in place of the primary and secondary antibodies, respectively.

Assay of Collagenase Activity The conditioned media were harvested and centrifuged at 9000 $\times g$ for 10 min at 4 °C. The supernatants (50 μ l each) were subjected to the assay of collagenase activity. Collagenase activity was determined fluorometrically with FITC (fluorescein isothiocyanate)-labeled type IV collagen as a substrate (the collagen assay kit was purchased from CosmoBio. Co., Tokyo, Japan).

Statistical Analysis of Data All values are expressed as the means $\pm$ S.D. (as indicated), and the significance level between groups was assessed by Student's *t* test.

RESULTS

Effects of NF and OSF on Tube Formation by HUVEC

One day after seeding, HUVEC formed capillary-like networks on Matrigel (Fig. 1A). In the presence of 25 μ g/ml OSF, however, very few complete network formations occurred (Fig. 1B). Tube formation by HUVEC was quantitatively estimated by measuring the tube length of the capillary-like networks of four microscopic fields (Fig. 2). OSF at 10–25 μ g/ml significantly inhibited the tube formation by 42–51%, and increasing the dose of OSF did not cause further inhibition. At the same concentration range, NF had little or no effect on the tube formation.

Effects of NF and OSF on Attachment and Migration of HUVEC As shown in Fig. 3, both fucoidans at 1–25 μ g/ml enhanced the attachment of HUVEC to Matrigel. Maximal adhesion by HUVEC was observed at a con-

Fig. 1. Light Micrographs of Capillary-Like Network Formation by HUVEC

(A) Control HUVEC; (B) OSF (25 μ g/ml)-treated HUVEC.

Fig. 2. Quantitative Determination of Tube Formation by HUVEC

HUVEC (3.5×10^3 cells/well) were incubated for 24 h on Matrigel with or without fucoidan. The total length of capillary networks formed were determined as described in Materials and Methods. Each bar represents the mean $\pm$ S.D. ($n=3$). Values significantly different from control: * $p<0.05$; ** $p<0.001$.

Fig. 3. Effects of NF and OSF on the Attachment of HUVEC to Matrigel

HUVEC were seeded at 5×10^3 cells/well, and the attached cells were counted 1 h after seeding. Each bar represents the mean $\pm$ S.D. ($n=4$). Values significantly different from control: * $p<0.05$.

centration of 10 $\mu\text{g/ml}$ for each fucoidan (NF, 133% of control; OSF, 147% of control, $p<0.05$). The addition of 50 $\mu\text{g/ml}$ each fucoidan decreased the HUVEC attachment to the control level (data not shown). We next examined the effects of NF and OSF on the migration of HUVEC as described in Materials and Methods. When NF was added to the lower chambers at the concentration range of 1–25 $\mu\text{g/ml}$, it had little or no effect on the migration of HUVEC (Fig. 4). By contrast, OSF at 1–10 $\mu\text{g/ml}$ significantly decreased the migration of HUVEC to the lower side of filter, by 80% of control ($p<0.05$). The addition of 25 $\mu\text{g/ml}$ OSF reduced the HUVEC migration by 40% of control ($p<0.001$). Increasing the concentration of OSF (50 $\mu\text{g/ml}$) did not increase the inhibitory effect (data not shown).

Effects of NF and OSF on t-PA and PAI-1 Secretion
Endothelial cell migration is closely correlated with the cellular level of PA activity.^{8,9)} HUVEC secreted t-PA antigen into the media at the concentration of 1.8 ± 0.09 ng/ml, as determined at 24 h (Fig. 5A). Neither NF nor OSF at 10–25 $\mu\text{g/ml}$ influenced the secretion of t-PA antigen from HUVEC. By contrast, the incubation of HUVEC with 10–25 $\mu\text{g/ml}$ OSF resulted in a 2.2 to 2.7-fold increase ($p<0.001$) in the PAI-1 antigen level, compared with the control (14.7 ± 1.34 ng/ml) (Fig. 5B).

Fig. 4. Effects of NF and OSF on the Migration of HUVEC

HUVEC (10^4 cells) were incubated for 2 d on the filter of a Transwell chamber, and the cells which migrated to the membrane pores were counted as described in Materials and Methods. Each bar represents the mean $\pm$ S.D. ($n=3$). Values significantly different from control: * $p<0.05$; ** $p<0.001$.

Fig. 5. Effects of NF and OSF on the Secretion of t-PA (Panel A) and PAI-1 (Panel B) Antigens from HUVEC

HUVEC were incubated for 24 h on Matrigel with or without fucoidan. The contents of t-PA and PAI-1 antigens in the medium were measured by ELISA. Each bar represents the mean $\pm$ S.D. ($n=3$). Values significantly different from control: ** $p<0.001$.

At a 6-h incubation of HUVEC with 25 $\mu\text{g/ml}$ OSF, the PAI-1 antigen level (17.0 ± 1.21 ng/ml) was already 2-fold higher than the control level (8.3 ± 0.84 ng/ml) (data not shown). On the other hand, the effect of NF on the PAI-1 antigen secretion by HUVEC was negligible.

Effects of NF and OSF on the Level of Collagenase Activity in Medium Basement membrane collagenases facilitate tube formation by endothelial cells, and their endogenous inhibitors, TIMPs, decrease the tube forming activity.¹⁰⁾ We therefore determined the collagenase (type IV) activity in the media from HUVEC that were incubated in Matrigel for 24 h in the presence or absence of fucoidan. As shown in Fig. 6, the collagenase activity level in the medium did not change by the presence of

Fig. 6. Effects of NF and OSF on the Level of Collagenase Activity in Medium

HUVEC were incubated for 24 h on Matrigel with or without fucoidan. The collagenase activity was measured with FITC-collagen as the substrate. Each bar represents the mean $\pm$ S.D. ($n=3$). Values significantly different from control: ** $p < 0.001$.

Fig. 7. Effects of NF and OSF on the TIMP-1 Antigen Level in Medium

HUVEC were incubated for 24 h on Matrigel with or without fucoidan. The conditioned media were harvested and subjected to Western blot analysis as described in Materials and Methods. Lanes 1 and 2, control medium; lanes 3–5, medium in the presence of 25 µg/ml NF; lanes 6–8, medium in the presence of 25 µg/ml OSF. Each medium was obtained from different experiments.

10–25 µg/ml NF. However, the presence of 10–25 µg/ml OSF significantly decreased the collagenase activity by 50–63% of the control level (0.145 ± 0.007 units/ml, $p < 0.001$). Figure 7 shows the TIMP-1 antigen level in the media, as analyzed by Western blotting. The result indicates that the incubation of HUVEC with NF or OSF (25 µg/ml) for 24 h does not affect the secretion of TIMP-1 antigen from HUVEC. We further examined the direct effect of fucoidan on the activity of type IV collagenase, purified from *Clostridium histolyticum*. In the purified system, neither NF nor OSF at 25 µg/ml exhibited enzyme inhibition (data not shown).

DISCUSSION

Using an *in vitro* angiogenic model and morphometric measurement, we have shown that OSF, but not NF, has the ability to suppress capillary-like network formation by HUVEC (Figs. 1 and 2). The assay system used in this study involves crucial steps that take place during angiogenesis *in vivo*. Matrigel that was used as the re-constituted basement membrane is derived from murine Engelbreth-Holm-Swarm sarcoma and contains several growth factors, such as epidermal growth factor (EGF), laminin and type IV collagen. These factors present in Matrigel may play important roles during the angiogenesis by HUVEC.

The migration of endothelial cells is a critical process in angiogenesis. EGF stimulates the migration of human

microvascular endothelial cells by increasing the production of t-PA.⁹⁾ t-PA can convert pro-collagenase to active collagenase through converting plasminogen to plasmin. Plasmin itself is also active in degrading extracellular components such as laminin and fibronectin. Endothelial cells produce high levels of basic fibroblast growth factor (bFGF),^{11–13)} and it subsequently promotes endothelial cell migration by increasing the PA production.¹⁴⁾ In this assay system, therefore, EGF and bFGF seem to be growth factors which contribute to the stimulation of HUVEC migration. OSF more strongly inhibited the HUVEC migration than NF (Fig. 4). The inhibitory activity of OSF may be closely related to its increased polyanionic nature compared with that of NF. Suramin is a polysulphonated naphthylurea that blocks angiogenesis.²⁾ It inhibits the binding of growth factors to their receptors on cells. EGF-induced endothelial cell migration was completely inhibited by this compound.⁹⁾ It is likely that OSF acts against growth factors, such as EGF and bFGF, and their receptors on HUVEC in a similar manner to suramin. If this were the case, OSF could decrease the PA secretion from HUVEC. However, a 24-h incubation of HUVEC with OSF did not influence the t-PA antigen level in medium (Fig. 5A). Yet, the possibility that OSF inhibits the interaction of EGF (or bFGF) with HUVEC in a mode different from that of suramin still remains.

A striking feature of OSF is that, unlike NF and other anionic polymers, it stimulates the secretion of PAI-1 antigen from HUVEC (Fig. 5B). Tumor necrosis factor- α (TNF- α)⁹⁾ and transforming growth factor- β (TGF- β)¹⁵⁾ which could be secreted by HUVEC, stimulate the synthesis and secretion of PAI-1 by human microvascular endothelial cells. These cytokines may be closely correlated with endothelial cell migration through the regulation of PA activity. The mechanism by which OSF, but not NF, increased the secretion of PAI-1 antigen from HUVEC is not known. One possibility is that OSF increased the PAI-1 secretion by increasing the secretion of TNF- α and/or TGF- β from HUVEC, and another is that OSF promoted the binding of these cytokines to their receptors or stimulated directly the PAI-1 secretion in a mode different from those of the cytokines. We are currently investigating these hypotheses and why OSF acted more effectively than NF.

The above results suggest that the balance of plasminogen activation and its inhibition shifts to inhibition with OSF. The predominance of the PAI-1 level in a medium may reduce plasmin generation, thereby reducing the conversion of pro-collagenases to the active enzymes. Indeed, incubation of HUVEC with OSF significantly decreased the collagenase activity level in medium by 50–63% of the control level (Fig. 6). The collagenase activity is regulated by TIMPs. OSF did not influence the medium level of TIMP-1 antigen that is secreted from HUVEC (Fig. 7). Additionally, OSF had no directly inhibitory effect on purified collagenase (data not shown). Whether OSF reduces the pro-collagenase secretion from HUVEC remains to be determined. However, these results indicate that OSF could inhibit endothelial cell migration and tube formation by reducing the potency of the PA-plasmin-collagenase system that

degrades basement membrane components. Finally, in angiogenesis, the detachment of endothelial cells from the basement membrane is important for the next process, cell migration. The ability of OSF to enhance the endothelial cell attachment to the basement membrane (that is, to inhibit the detachment of cells from the membrane) (Fig. 3) may partly contribute to its anti-angiogenic property. The mechanism(s) and *in vivo* effect of OSF will need to be explored further in comparison with those of other polyanionic agents such as suramin.

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