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Inhibitory Effect of Oversulfated Fucoidan on Invasion through Reconstituted Basement Membrane by Murine Lewis Lung Carcinoma

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We investigated the effects of native, oversulfated, and desulfated fucoidans and heparin on the invasion of 3 LL cells through Matrigel. Of the four polysaccharides tested, oversulfated fucoidan was the most potent inhibitor of tumor cell invasion and inhibited most potently and specifically the tumor cell adhesion to laminin. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of the binding of elastase-cleaved laminin to fucoidan- and heparin-Sepharoses showed that both polysaccharides bound to the 62 and 56 kDa fragments. Pretreatment of 3LL cells with native or oversulfated fucoidan reduced their adhesive potency to laminin. The two fucoidans inhibited further the laminin binding of 3 LL cells which had been pretreated with a laminin-based pentapeptide, YIGSR. These results suggest that fucoidan specifically binds to not only the heparin binding domain(s) of laminin but also site(s) other than the cell surface laminin receptor. 3 LL cells secreted a 50 kDa form of urokinase-type plasminogen activator (u-PA). The extracellular level of u-PA activity was increased 1.7 times by addition of laminin but not type IV collagen. Oversulfated fucoidan most potently reduced the increased u-PA levels. Therefore, the reduction in *in vitro* invasiveness of 3 LL cells in response to either fucoidan or its oversulfated derivative may result from an inhibition of physical interaction between the tumor cells and the Matrigel (laminin), followed by a suppression of the laminin-induced increase in extracellular u-PA.

Key words: Lewis lung carcinoma — Invasion — Laminin — Urokinase — Fucoidan

The invasion of tumor cells through basement membranes is a crucial step in the formation of metastasis. The binding of the malignant cells to matrix proteins in the basement membrane allows their attachment and activates their invasiveness. However, the precise mechanism of metastasis has not yet been fully elucidated. Several lines of evidence indicate that an increased production of proteolytic enzymes is associated with the invasive and/or metastatic potential of many malignant cells. Namely, u-PA[2] secreted by tumor cells converts the abundant proenzyme, plasminogen, into plasmin. Plasmin degrades noncollagenous matrix proteins, such as laminin and fibronectin, and activates procollagenase secreted from the tumor cells themselves.[1,2]

Several sulfated polysaccharides have been experimentally used as antimetastatic agents.[3] The basis for this use is the assumption that the polysaccharides inhibit the formation of tumor emboli caused by platelet aggregation at the early stage of tumor lodgment. However, chemically modified heparins without anticoagulant activity also reduce the number of experimental lung metastases of melanoma cells by inhibiting the activity of heparanase secreted from these cells.[4] Sulfated chitin derivatives play an inhibitory role in matrix degradation by melanoma cells.[5] Thus, studies addressing the role of such sulfated polysaccharides in tumor cell invasion and metastasis still continue.

Fucoidan is a sulfated poly(L-fucopyranose) present in brown marine algae and has been reported previously to have antimetastatic activity.[6] In this study, we focused our attention on the tumor invasion process and examined the effects of fucoidan and its oversulfated derivative on Lewis lung carcinoma (3 LL cell) invasion through reconsituted membrane. The results suggest that oversulfated fucoidan potently reduces the tumor cell invasion by suppressing the laminin-induced increase in extracellular u-PA.

MATERIALS AND METHODS

Cell culture 3 LL cells were purchased from the RIKEN Cell Bank, Tsukuba. The cells were grown in RPMI 1640 (GIBCO) supplemented with 10% heat-inactivated (60°C, 30 min) fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C in a humidified 5% CO_2 atmosphere. Adherent cells were detached with

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[2] The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoretic; u-PA, urokinase-type plasminogen activator; PBS, phosphate-buffered saline; TPBS, PBS/Tween 20; EHS, Engelbreth-Holm-Swarm; HPF, high-power field; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; YIGSR, Try-Ile-Gly-Ser-Arg; Glt-Gly-Arg-MCA, glutaryl-glycyl-L-arginine 4-methylcoumaryl-7-amide; AMC, 7-amino-4-methylcoumarin.

0.05% EDTA in 20 m*M* PBS and harvested by centrifugation.

Invasion assay The invasive activity of 3 LL cells was assayed according to the method of Albini *et al.*[7] with some modifications.[8] Briefly, 8-μm polycarbonate filters (Nucleopore, Pleasanton, CA) were coated with 50 μg of Matrigel (Collaborative Research, Inc., Bedford, MA) and placed in a modified Boyden blind well chamber (NeuroProbe, Inc., Cabin John, MD). Conditioned medium (200 μl), obtained by incubating NIH 3T3 cells (RIKEN) for 24 h in serum-free RPMI 1640 medium containing 50 μg/ml of ascorbate, was placed in the lower compartment of the Boyden chamber. 3 LL cells (3×10^5 cells/0.8 ml) were added to the upper chamber and incubated at 37°C for 6 h in a humidified 5% CO_2 atmosphere. The tumor cells that remained on the upper surface of the filter were mechanically removed. The cells on the reverse surface of the filter were fixed and stained with Diffi-Quik (American Scientific Products, McGaw Park, IL). The stained cells were counted in 9 microscope fields ($\times$200 HPF).

Adhesion assay Murine EHS tumor-derived laminin and type IV collagen and murine plasma fibronectin (CosmoBio., Tokyo) were diluted with PBS at the concentrations of 25 μg/ml, 100 μg/ml, and 25 μg/ml, respectively, and 200 μl of each solution was incubated in 96-well plates at 4°C overnight. The wells were thereafter treated with 0.1% BSA in PBS at 25°C for 2 h, and washed 3 times with 200 μl of PBS. To each well were added 50 μl of polysaccharide solution (1–4 μg/ml in RPMI 1640 containing 0.1% BSA) and 50 μl of the cell suspension (1×10^5/ml in RPMI 1640 containing 0.1% BSA). The cells were allowed to adhere at 37°C in a humidified 5% CO_2 atmosphere. After 60 min, unbound cells were removed by washing the wells with 100 μl of PBS. The adherent cells were fixed with 2.5% glutaraldehyde solution for 10 min and stained with Giemsa's stain (Merck). The stained cells were microscopically counted (5 fields; $\times$100 HPF).

Preparation of fucoidan derivatives Native fucoidan (200 mg, Sigma) was purified on a Sephadex G-100 column (2.5 $\times$ 36 cm) equilibrated with 0.5 *M* NaCl. Oversulfated fucoidan with a molecular mass of 100–130 kDa was prepared as described previously.[9] Briefly, the purified fucoidan was further sulfated in a mixture of dimethylformamide and sulfur trioxide-trimethylamine complex at 50°C for 24 h. The product recovered by ethanol precipitation was purified on a Sephadex G-100 column. The yield of oversulfated fucoidan was 144 mg, and the sulfate content was estimated to be 52.4% (for the native form, 31.2%). Desulfated fucoidan was prepared by treating the purified fucoidan with 10% (v/v) methanol in dimethyl sulfoxide at 80°C for 18 h.[9] Biotin-labeled fucoidan was prepared as follows. To introduce a spacer ($-O-CH_2CH(O)CH_2$), the purified fucoidan (50 mg) was dissolved in 750 μl of water and mixed with 250 μl of 2.6 *N* NaOH and 150 μl of epichlorohydrin. The mixture was stirred at 40°C for 2 h and dialyzed against water at 25°C for 2 days. After lyophilization of the dialysate, the residue was dissolved in 3 ml of 30% ammonia water and aminated at 40°C for 90 min. The reaction mixture was dialyzed against water and lyophilized. Twenty mg of the aminated product was dissolved in 5 ml of 0.1 *M* $NaHCO_3$ (pH 8.4) and mixed with 200 μl of a biotinyl-N-hydroxysuccinimide ester solution (1 mg/ml in dimethylsulfoxide, Sigma). The mixture was stirred at 25°C for 2 h and dialyzed against water at 4°C overnight. The dialysate (biotinylated fucoidan) was stored at -20°C until use.

ELISA assay Laminin was dissolved in PBS at a concentration of 5 μg/ml, and 200 μl of the solution was incubated in 96-well plates at 4°C overnight. The wells were washed 5 times with 200 μl of TPBS and coated with 200 μl of 1% BSA in PBS at 4°C overnight. The wells were washed exhaustively with TPBS, and various amounts of biotin-labeled fucoidan (200 μl) were added. The plates were incubated at 25°C for 4 h and washed 5 times with 200 μl of TPBS. To detect the bound fucoidan, 200 μl of a solution of streptavidin-peroxidase conjugate (Bethesda Research Lab., Inc., Gaithersburg, MD) diluted 1:1000 in PBS was added to each well and incubation followed at 25°C for 1 h. The wells were exhaustively washed with TPBS, and the activity of bound peroxidase was measured with *o*-phenylenediamine as the substrate.

Determination of u-PA activity 3 LL cells (1.5×10^6) suspended in 40 ml of the RPMI 1640 medium containing 10% heat-inactivated serum were cultured at 37°C in a 175-cm^2 tissue culture flask. For testing the effect of matrix protein on u-PA secretion from 3 LL cells, the medium contained laminin, fibronectin or type IV collagen at a concentration of 30 μg/ml and, if indicated, oversulfated fucoidan or heparin at 10 μg/ml. YIGSR was added to the medium at a concentration of 10 μg/ml. After cell culture for 6 h, each medium was collected and concentrated to a volume of 2 ml by the use of Centricon-10 concentrators (Amicon, Inc., Beverly, MA). Pro-u-PA in the concentrated medium was treated at 37°C for 30 min with a catalytic amount of plasmin (0.01 CU/ml), and then the remaining plasmin activity was neutralized by adding aprotinin (5 μg/ml). Amidolytic u-PA activity was measured with Glt-Gly-Arg-MCA (Peptide Institute, Inc., Osaka). The assay mixture contained 200 μl of the concentrated medium, 0.2 m*M* substrate and 50 m*M* Tris-HCl buffer (pH 8.0) in a total volume of 1.7 ml. After incubation of the mixture at 37°C overnight, the reaction was stopped by adding 2.5 ml of 17% acetic acid solution. The fluorescence of AMC released was mea-

sured by the use of a Hitachi 650-10 S fluorophotometer with excitation at 380 nm and emission at 460 nm. Zymographic analysis of u-PA in the medium was performed by the method of Tissot *et al.*[10] In this experiment, 20 μl of the concentrated medium was used.

Affinity chromatography Fucoidan and heparin (Sigma) were coupled to epoxy-activated Sepharose 6B (Pharmacia) according to the manufacturer's procedure. The washed gels contained ~20 μg of polysaccharide per ml of gel. Laminin (0.5 mg) was dissolved in 250 μl of 50 m*M* Tris-HCl buffer (pH 7.4) containing 0.15 *M* NaCl and digested at 37°C for 20 h with 2.0 units of porcine pancreas elastase (Boehringer). The digested laminin was applied to a fucoidan- or heparin-Sepharose column (0.8-ml bed volume) equilibrated with 50 m*M* Tris-HCl buffer (pH 7.4) containing 0.15 *M* NaCl, and the column was washed with the equilibration buffer. The bound proteins were eluted with 1.5 ml of 50 m*M* Tris-HCl buffer (pH 7.4) containing 1 *M* NaCl. The fractions passed-through and eluted from the column were concentrated with Centricon-10, and aliquots (15 μl) of the concentrates were subjected to 12% (w/v) SDS-PAGE under reducing conditions.[11] The gel was stained with Brilliant Blue R (Sigma) to detect the protein fragments. Molecular size standards were the product of Promega, Madison, WI.

RESULTS

Inhibition of 3 LL cell invasion by native, oversulfated and desulfated fucoidans and heparin We first examined the effects of these polysaccharides on the invasion of 3 LL cells through a reconstituted basement membrane, Matrigel. As shown in Fig. 1, native fucoidan, its oversulfated derivative and heparin inhibited the tumor cell invasion in a concentration-dependent manner. Among them, oversulfated fucoidan showed the highest inhibitory effect (90% inhibition at 30 μg/ml; half-maximal inhibition at 5 μg/ml). However, desulfated fucoidan had no inhibitory effect. In addition, the viability and growth of 3 LL cells were not affected even by exposure to 20 μg/ml of oversulfated fucoidan for 3 days (data not shown), indicating that the inhibitory potency of this polysaccharide is independent of its cytotoxicity.

Effects of fucoidan, its derivatives and heparin on the tumor cell adhesion We next examined the effects of these polysaccharides on the attachment of 3 LL cells to laminin, fibronectin or type IV collagen (Fig. 2). As shown in panel A, native and oversulfated fucoidans effectively inhibited the tumor cell adhesion to laminin in a concentration-dependent manner from 0 to 2 μg/ml, with half-maximal inhibitions being obtained at 1.5 and 0.5 μg/ml, respectively. However, the inhibitory effects of both heparin and desulfated fucoidan were negligible.

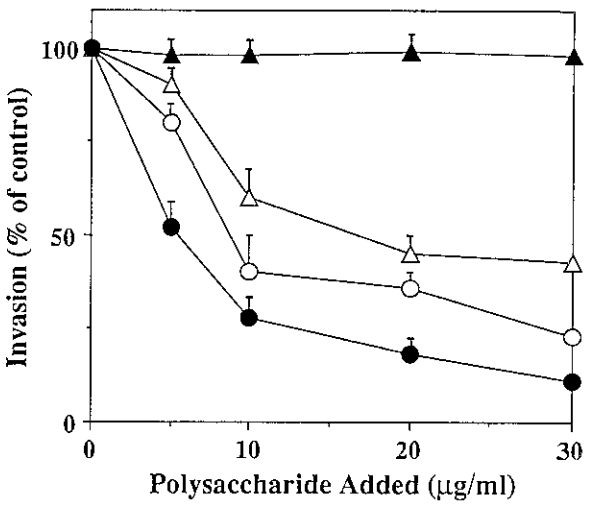

Fig. 1. Inhibition of 3 LL cell invasion through Matrigel by native fucoidan (○), oversulfated fucoidan (●), desulfated fucoidan (▲), and heparin (△). Experimental procedures are as described in "Materials and Methods." Results are presented as percent of the control cells normalized to 100% in the absence of polysaccharide (mean±SD, n=3).

On the other hand, these polysaccharides had little or no inhibitory effect on the tumor cell adhesion to fibronectin (panel B) or type IV collagen (panel C).

Fucoidan binding to laminin To investigate further the laminin selectivity of fucoidan, we prepared biotin-labeled fucoidan and examined its binding ability to laminin-coated wells. As shown in Fig. 3A, the fucoidan derivative (1–100 ng/well) bound to laminin in a concentration-dependent manner. Additionally, the fucoidan binding was prevented by pretreatment of the laminin-coated wells with an excess amount (1 μg/well) of heparin. Biotinylated fucoidan did not bind to the immobilized BSA used as a reference. To localize the fucoidan-binding region(s) of laminin, its elastase-cleaved products were prepared and chromatographed on a fucoidan- or heparin-Sepharose column. SDS-PAGE analysis (Fig. 3B) indicated that the digestion of laminin with elastase yielded many fragments with molecular masses of 23–75 kDa (lane 2). Laminin fragments that bound to both fucoidan- and heparin-Sepharose columns were detected as 56 and 62 kDa bands (lanes 4 and 6). The high-molecular-weight (> 97 kDa) laminin fragment(s) seen in lane 2 did not bind to heparin-Sepharose (lane 5). However, it might be tightly adsorbed to fucoidan-Sepharose, because neither the passed-through fraction (lane 3) nor the eluted fraction (lane 4) contained the band.

Fucoidan binding to the tumor cell surface In this experiment, 3 LL cells were preincubated at 37°C with 0–2

μg/ml of fucoidan, oversulfated fucoidan or heparin. The cells were washed extensively, and their adhesion to laminin-coated wells was examined. As shown in Fig. 4, these polysaccharides inhibited the tumor cell adhesion in a concentration-dependent manner, and oversulfated fucoidan was the most potent inhibitor (50% inhibition at 2 μg/ml). We next examined the effects of these sulfated polysaccharides on the laminin attachment of 3 LL cells, pretreated with the synthetic laminin B1 chain peptide, YIGSR. The adhesion of 3 LL cells to laminin was inhibited by pretreatment of the cells with various concentrations of YIGSR: maximal inhibition (20%) was

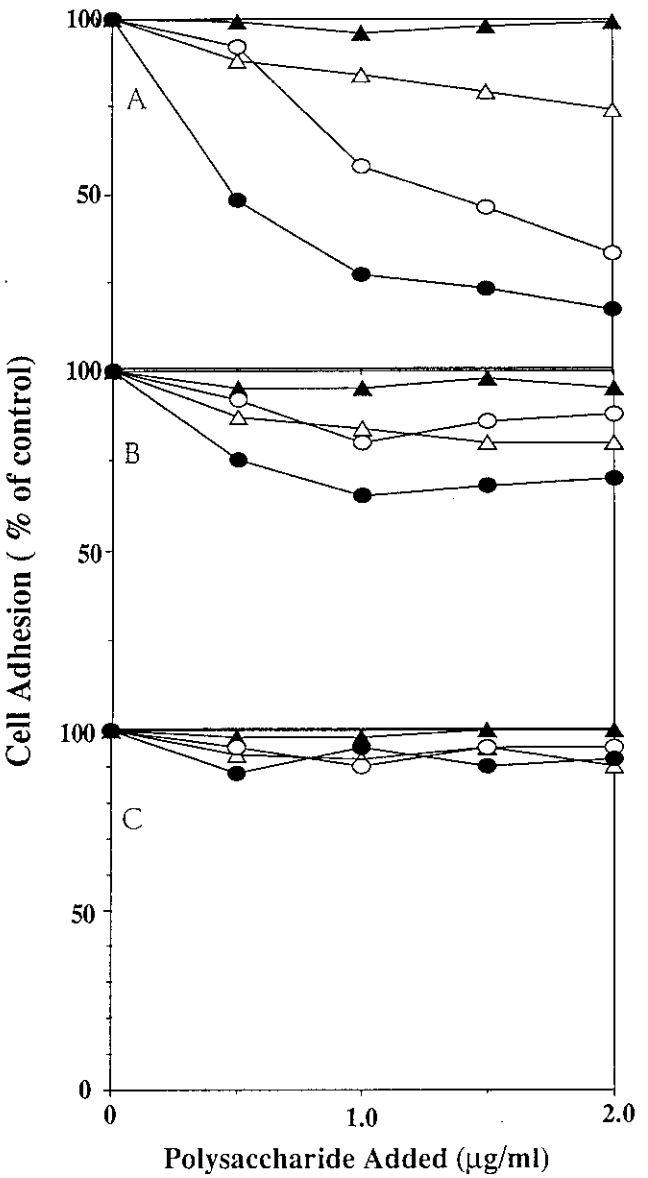

Fig. 2. Effects of polysaccharides on 3 LL cell adhesion to laminin (panel A)-, fibronectin (panel B)-, or type IV collagen (panel C)-coated wells. 3 LL cells (5×10^3) were added to the substrate with or without polysaccharide: (○), native fucoidan; (●), oversulfated fucoidan; (▲), desulfated fucoidan; or (△), heparin. After a 2-h incubation, unbound cells were washed away, and the attached cells were counted. Results are presented as percent of the control cells normalized to 100% in the absence of polysaccharide (mean±SD, n=3).

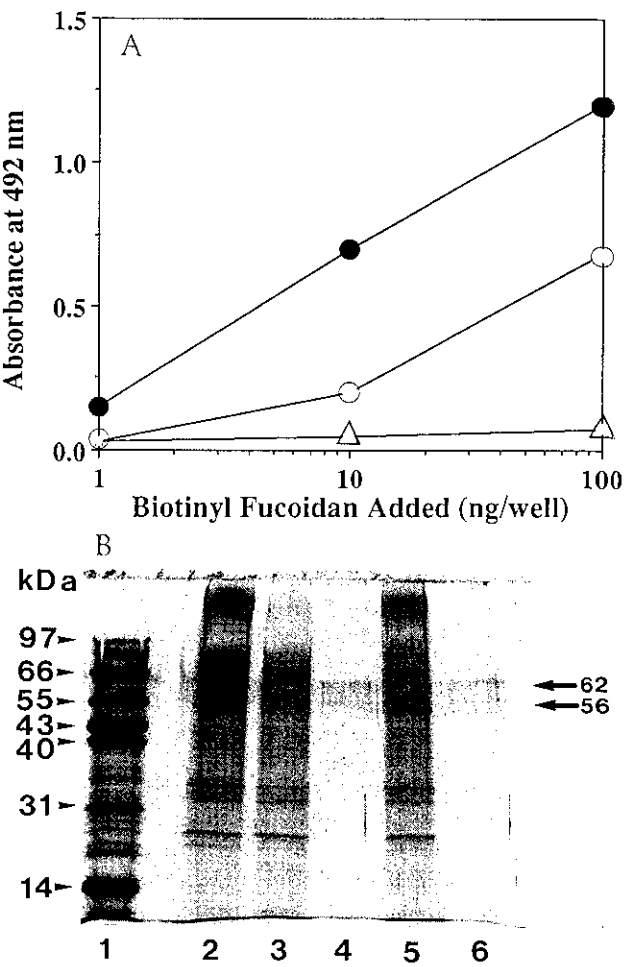

Fig. 3. Fucoidan binding to laminin. Panel A: concentration-dependent binding of biotin-labeled fucoidan to laminin substrate in the absence (●) or presence (○) of heparin (1 μg/well). BSA-coated wells were used as the control (△). Each point is the mean of duplicate experiments. Panel B: SDS-PAGE analysis of the binding of elastase-digested laminin to a fucoidan- or heparin-Sepharose column. Lane 1, molecular weight standards; lane 2, laminin fragments before chromatography; lane 3, laminin fragments passed through fucoidan-Sepharose; lane 4, laminin fragments eluted from fucoidan-Sepharose; lane 5, laminin fragments passed through heparin-Sepharose; lane 6, laminin fragments eluted from heparin-Sepharose.

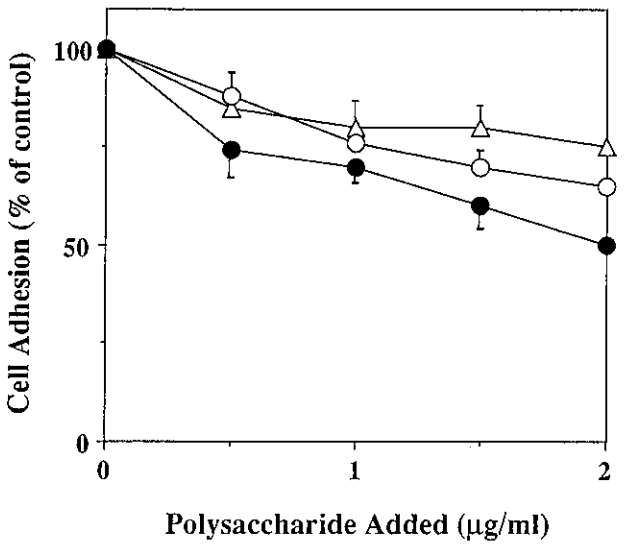

Fig. 4. The laminin attachment of 3 LL cells pretreated with various concentrations of native fucoidan (○), oversulfated fucoidan (●) or heparin (△). 3 LL cells (5×10^3) were preincubated at 37°C for 1 h with 0–2 μg/ml of each polysaccharide, and the polysaccharide bound nonspecifically to the cells was washed away with the conditioned medium. The cells were allowed to adhere to the laminin-coated wells as described in "Materials and Methods." Results are presented as percent of the control cells normalized to 100% in the absence of polysaccharide (mean±SD, n=3).

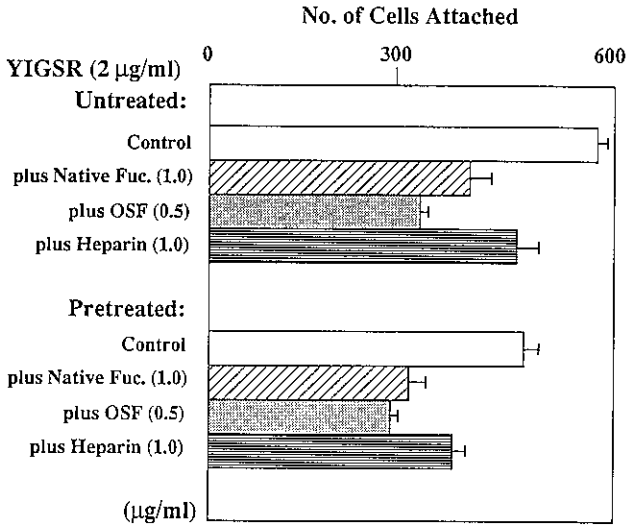

Fig. 5. Effects of polysaccharides on the laminin attachment of 3 LL cells pretreated with YIGSR. 3 LL cells (5×10^3) were incubated at 37°C for 1 h with 2 μg/ml of YIGSR, washed with the conditioned medium, and seeded into laminin-coated wells with or without the indicated polysaccharide. Cell adhesion was assayed as described in "Materials and Methods." Each bar is the mean±SD (n=3).

obtained with 1–2 μg/ml (data not shown). As shown in Fig. 5, the inhibitory effects of native fucoidan (1 μg/ml), oversulfated fucoidan (0.5 μg/ml) and heparin (1 μg/ml) on the laminin attachment of YIGSR (2 μg/ml)-pretreated 3 LL cells were almost the same as those on the attachment of nontreated cells.

Effects of matrix proteins and sulfated polysaccharides on extracellular u-PA levels We tested the effect of laminin (major component of Matrigel) on the extracellular u-PA levels using fibrin zymography technique. As shown in Fig. 6, 3 LL cells grown for 6 h in the conditioned medium alone (lane A) and in the medium supplemented with 30 μg/ml of laminin (lane B) or 30 μg/ml of laminin plus 10 μg/ml of oversulfated fucoidan (lane C) secreted plasminogen activator, which was detected as a 50 kDa lysis band. The fibrinolytic protein band was recognized with anti-human u-PA IgG (data not shown). The result also suggested that 3 LL cells increased the extracellular u-PA level in the presence of laminin (lane B) and that the increase of u-PA was suppressed by the simultaneous presence of oversulfated fucoidan (lane C). To confirm this, we next measured the extracellular u-PA level of 3 LL cell culture using Glt-Gly-Arg-MCA as the substrate. As shown in Fig 7A, the

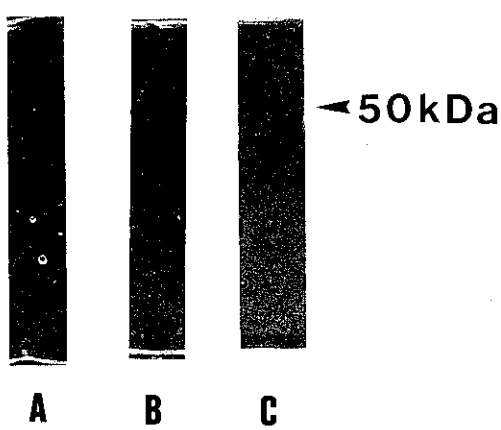

Fig. 6. Semi-quantitative determination of u-PA-related fibrinolytic activity in the conditioned medium. 3 LL cells (1.5×10^6) were cultured for 6 h in the absence (A) and presence of 30 μg/ml of laminin (B) or 30 μg/ml of laminin plus 10 μg/ml of oversulfated fucoidan (C). Twenty μl of the concentrated medium was applied to 13% SDS-polyacrylamide gel. After electrophoresis, the gel was incubated at 25°C for 1 h with 2.5% (v/v) Triton X-100 solution and then at 37°C for 24 h on a plasminogen-rich fibrin plate.

activity of u-PA secreted from 3 LL cells adherent to laminin was 1.7 times that (control) from the cells adherent to plastic surfaces. Oversulfated fucoidan (10 μg/ml)

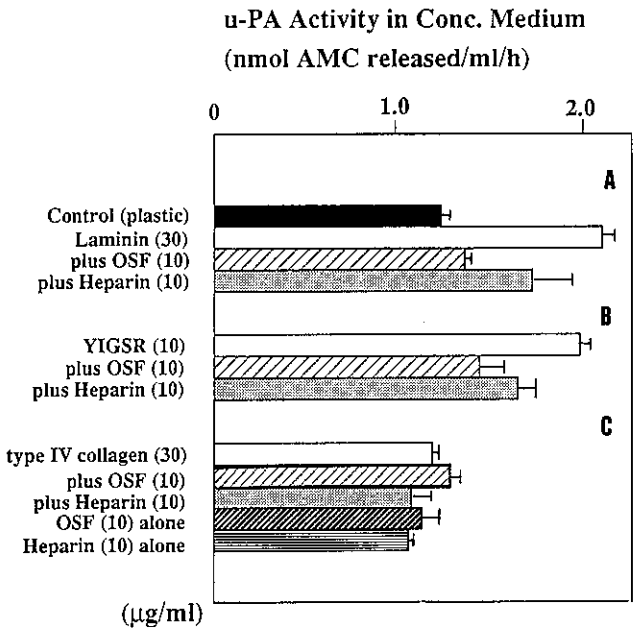

Fig. 7. Effect of matrix proteins and sulfated polysaccharides on extracellular u-PA level. Experimental procedures are as described in "Materials and Methods." Each bar is the mean±SD (n=3).

suppressed the laminin-enhanced increase in extracellular u-PA by 90%. However, the suppressive effect of heparin at a concentration of 10 μg/ml was lower than that of oversulfated fucoidan. As shown in panel B, addition of YIGSR (10 μg/ml) induced a 1.6-fold increase in the extracellular u-PA. The increase of u-PA was suppressed by addition of oversulfated fucoidan (120% of control). Neither type IV collagen (30 μg/ml) alone nor the collagen (30 μg/ml) plus polysaccharide (10 μg/ml) changed the u-PA level. Oversulfated fucoidan or heparin alone had no effect on the u-PA secretion (panel C).

DISCUSSION

Several fucoidan derivatives were prepared by chemical sulfation and desulfation, and examined for the ability to suppress the invasion of 3 LL cells through Matrigel (Fig. 1). The magnitude of their activities was dependent upon the degree of sulfation. The abilities of fucoidan derivatives specifically to reduce the tumor cell adhesion to laminin also depended on the degree of sulfation (Fig. 2). The most potent inhibitor, oversulfated fucoidan, has sulfate groups on both the C_3- and C_4-positions of the fucose unit. Therefore, a particular spatial orientation of the negative charge(s) in the fucoidan molecule may also be an important determinant.

Fucoidan and heparin bound to the 62 and 56 kDa fragments of elastase-digested laminin (Fig. 3B). An ELISA assay showed a competitive binding of fucoidan and heparin to immobilized laminin (Fig. 3A). Therefore, as a mechanism for the inhibitory effect of oversulfated fucoidan on the 3 LL cell invasion, it is conceivable that the polysaccharide selectively binds to the heparin-binding domain(s) of laminin in the reconstituted basement membrane and hence disturbs the attachment of 3 LL cells to the membrane. However, whether fucoidan and heparin bind to the same site(s) in the laminin molecule remains unknown.

Other data (Fig. 4) suggest that native and oversulfated fucoidans, as well as heparin, bind to the cell surfaces of 3 LL cells. Binding of heparin or heparan sulfate to a number of cell types has already been reported.[12-14] However, in most cases, the binding proteins have not been fully characterized. Recently, Biswas[15] has identified a 14 kDa heparin-binding protein on mouse B-16 melanoma cell membranes. Whether such binding proteins exist in 3 LL cells and function as cell surface receptors for fucoidan and related polysaccharides remains unclear at present. On the other hand, various cell surface components, including several integrins, β-galactoside and 67 kDa laminin receptor, have a high affinity for laminin.[16] The synthetic laminin pentapeptide, YIGSR, specifically binds to the 67 kDa receptor and inhibits experimental metastasis formation.[17] Our present study demonstrated that the laminin attachment of YIGSR-pretreated and non-treated 3 LL cells was equally inhibited by fucoidan, its oversulfated derivative or heparin (Fig. 5). Therefore, we could exclude at least the 67 kDa laminin receptor as the polysaccharide-binding site.

In the "three-step" invasion hypothesis,[18-20] the first step involves attachment of tumor cells to laminin in the basement membrane; during the next step, proteolytic enzymes from the tumor cells degrade the matrix components; and the third step involves their penetration through the basement membrane. Our present results (Fig. 2) clearly show that oversulfated fucoidan is effective on the first of these steps. As regards the second step, it has been reported that u-PA secreted from tumor cells can trigger matrix breakdown.[1,2] 3 LL cells secreted a 50 kDa form of u-PA (Fig. 6), in accordance with the finding of Skriver *et al.*[21] The attachment of the tumor cells to laminin but not to type IV collagen caused a 1.7-fold increase in the extracellular u-PA levels (Fig. 7). A similar effect was observed with YIGSR (1.6-fold increase). The latter finding suggests that the 67 kDa laminin receptor is the most likely candidate for regulating the synthesis and/or secretion of this protease. The secretion of u-PA from human colon tumor cells is induced by endogenous diglycerides,[22] suggesting protein

kinase C dependency. To our knowledge, no direct linkage between the 67 kDa laminin receptor and the protein kinase C pathway has yet been described. Recently, Massia *et al.*[23] suggested that the laminin receptor could be a transmembrane protein which associates with vinculin, α-actinin, and possibly other proteins to form a connection with the cytoskeleton. The laminin (YIGSR)-mediated clustering and cytoskeletal association may promote not only cell spreading, but also indirectly the synthesis and/or secretion of intracellular proteins such as u-PA. Further studies will be required to evaluate this possibility.

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