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FIBRINOLYTIC AND ANTICOAGULANT ACTIVITIES OF HIGHLY SULFATED FUCOIDAN

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Abstract—A series of fucoidan [sulfated poly(L-fucopyranose)] derivatives were prepared by chemical sulfation and desulfation, and they were tested for their abilities to stimulate tissue plasminogen activator (t-PA)-catalyzed plasminogen activation, clot lysis, and the inhibition of fibrin polymer formation. The magnitude of their activities was dependent upon the degree of sulfation. A striking feature of the sulfated fucoidan was that, unlike heparin, it stimulated t-PA-induced plasma clot lysis by protecting plasmin activity from α_2-plasmin inhibitor and decreased the rate of fibrin polymer formation. The inhibition of hyaluronic acid-mediated enhancement of fibrin clot formation was also observed with the fucoidan derivative. We also showed that highly sulfated fucoidan prevents significantly endotoxin-induced hepatic vein thrombosis in the hyperlipemic rat model. The present results are the first to describe the fibrinolytic and anticoagulant activities of fucoidan, and thus may provide useful clues for the development of an ideal thrombolytic agent.

Considerable attention has been focused recently on the therapeutic value of tissue plasminogen activator (t-PA†) as a thrombolytic agent [1]. Although t-PA activates preferentially fibrinbound plasminogen with the potential of sparing fibrinogen, one possible problem of t-PA therapy is the rapid reocclusion of the affected artery. Therefore, patients treated with t-PA are treated concomitantly with an anticoagulant, heparin, to prevent ongoing coronary thrombosis or reocclusion after initially successful thrombolysis. However, some reports suggest that heparin cannot prevent coronary reocclusion in patients treated with t-PA [2, 3]. A possible explanation for the limited efficacy of heparin is that it promotes the binding of thrombin to fibrin polymer by forming a ternary complex [4], where the rate constant for inactivation of thrombin by heparin–antithrombin III (AT-III) is decreased 300-fold or more [5]. The altered reactivity of thrombin toward its substrate and inhibitor may be attributed to the growing of thrombus. Thus far, the search for an ideal anticoagulant that maximizes the efficacy of the thrombolytic agent and minimizes undesirable side effects still continues.

As a part of studies on the development of an ideal anticoagulant, we focused on fucoidan, a sulfated poly(L-fucopyranose), which is enriched in brown marine algas and has been reported previously to have anticoagulant activity [6]. Church *et al.* [7] have suggested recently that the major antithrombin activity of fucoidan is mediated by heparin cofactor II. In the present paper, we report that fucoidan and its derivatives stimulated t-PA-catalyzed plasminogen activation and prevented the formation of fibrin polymer. We also demonstrated that one of the fucoidan derivatives, highly sulfated fucoidan, is useful for the prevention of endotoxin-induced hepatic vein thrombosis in hyperlipemic rats.

MATERIALS AND METHODS

Reagents. The following reagents were commercially obtained: *Fucus vesiculosus* fucoidan, human umbilical cord hyaluronic acid and porcine intestinal mucosa heparin from the Sigma Chemical Co., St. Louis, MO, U.S.A.; *Salmonella typhosa* (0901, Westphal type) lipopolysaccharide from Difco Laboratories, Detroit, MI, U.S.A.; Sephadex G-75 from the Pharmacia Fine Chemical Co., Uppsala, Sweden; and *H*-D-valyl-L-leucyl-L-lysine *p*-nitroanilide (S-2251) from Kabi Vitrum AB, Stockholm, Sweden. All other reagents were of the best grade available from the Wako Pure Chemical Co., Osaka, Japan.

Purified proteins. Glu- and Lys-plasminogens were obtained from Kabi Vitrum AB and the Green Cross Co., Osaka, Japan, respectively. α_2-Plasmin inhibitor (α_2-PI) was purchased from Protogen AG, Laufelfingen, Switzerland. Recombinant human t-PA (TD-2061, 550,000 IU/mg protein) was supplied by the Daiichi Pharmaceutical Co., Tokyo, Japan. A stabilizing agent added to the t-PA preparation was removed by affinity chromatography on heparin–Sepharose [8]. Melanoma plasminogen activator inhibitor type 1 (PAI-1) was obtained from American Diagnostica Inc., Greenwich, CT, U.S.A., and reactivated by treatment with 4 M guanidinium

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† Abbreviations: t-PA, tissue plasminogen activator; Glu-plasminogen, native human plasminogen with NH_2-terminal glutamic acid (residues 1–790); Lys-plasminogen, human plasminogen with NH_2-terminal lysine (residues 77–790); α_2-PI, α_2-plasmin inhibitor; PAI-1, plasminogen activator inhibitor type 1; AT-III, antithrombin III; S-2251, *H*-D-valyl-L-leucyl-L-lysine *p*-nitroanilide; TPBS, Tween 20-containing phosphate-buffered saline; CU, casein unit; and DIC, disseminated intravascular coagulation.

chloride at pH 5.5 for 5 min. Plasminogen-rich human fibrinogen was obtained from Kabi Vitrum AB and rendered plasminogen-free by lysine–Sepharose chromatography [9]. Human thrombin (19 NIH units/mg protein) was obtained from the Boehringer Mannheim Yamanouchi Co., Osaka, Japan.

Sulfation of fucoidan. A highly O-sulfated derivative of fucoidan was prepared as follows. Fucoidan (200 mg) was dissolved in 5 mL of water and dialyzed exhaustively against water to remove low molecular weight materials. The dialysate was neutralized with tetrabutylammonium hydroxide and lyophilized. After drying the dialysate over P_2O_5 *in vacuo*, the residue was suspended in 10 mL of dry dimethylformamide. The suspension was mixed with sulfur trioxide–trimethylamine complex (667 mg) and allowed to react at 50° for 24 hr. After cooling, the reaction mixture was mixed with 2–3 mL of a saturated solution of sodium acetate in ethanol, and poured into cold ethanol. The precipitate formed (highly sulfated fucoidan) was collected by centrifugation, dissolved in 0.5 M NaCl, and purified by passing through a Sephadex G-75 column (2.5 × 42 cm) equilibrated with 0.5 M NaCl. The yield of highly sulfated fucoidan was 144 mg. Less extensive O-sulfation of fucoidan was achieved by using the technique of Horton and Just [10]. Fucoidan (100 mg) was suspended in 5 mL of dry pyridine and, to this, a chlorosulfonic acid–pyridine mixture (1:4, 2.5 mL) was added. After reflux on a steam bath for 90 min, the resultant solid product was collected by discarding the supernatant solution and dissolved in cold water. The solution was adjusted to pH 9 with 2 M NaOH. The precipitate formed by the addition of ethanol was dissolved in water and purified on the Sephadex G-75 column. The yield of mildly sulfated fucoidan was 56 mg.

Desulfation of fucoidan. O-Desulfated fucoidan was prepared by treating fucoidan (100 mg) with 15 mL of 10% (v/v) methanol in dimethyl sulfoxide (DMSO) at 80° for 18 hr. The desulfated product was recovered by ethanol precipitation and purified by Sephadex G-75 chromatography. The yield of desulfated fucoidan was 62 mg.

Determination of sulfate. The degree of sulfation or desulfation of fucoidan was estimated turbidimetrically as barium sulfate after HCl hydrolysis. Gelatin was used as a cloud-stabilizer and absorbance was measured at 360 nm [11].

Amidolytic assays. The stimulative effects of fucoidan and its derivatives on t-PA-catalyzed plasminogen activation were assayed by measuring their effects on the amidolytic activity caused by plasmin which was produced during the assay. The assay mixture contained 0.75 mM S-2251, 130 nM Lys-plasminogen, 300 IU/mL t-PA, 0–100 μg/mL fucoidan or its derivative, and 20 mM sodium phosphate buffer (pH 7.2)/0.15 M NaCl/0.05% (v/v) Tween 20 (20 mM TPBS) in a total volume of 200 μL. The mixture was incubated at 25° in 96-well plates for various periods of time, and the change in absorbance at 405 nm (A_{405}) was monitored by a plate reader (model MPR-A4, Toyo Soda, Tokyo, Japan). The initial rate of plasminogen activation was obtained from the slope of plots of absorbance change as a function of time squared. Kinetic analyses of the Lys-plasminogen activation in the presence and the absence of fucoidans were performed as described previously [12]. The inhibitory effect of PAI-1 on the plasmin generation by t-PA and the inhibitory effect of α_2-PI on the generated plasmin were determined as follows. The assay mixture contained 0.75 mM S-2251, 200 nM Glu-plasminogen, 0 to 0.6 μg/mL PAI-1 (or 0 to 6.5 μg/mL α_2-PI), 300 IU/mL t-PA, 20 mM TPBS, and, if indicated, 25 μg/mL fucoidan or its derivative in a total volume of 200 μL. The assay was started by the addition of t-PA, and the A_{405} was measured at 25° for 20 min using the plate reader.

Determination of clot lysis time. Human plasma (250 μL) was mixed at 4° with 50 μL of a t-PA solution (300 IU/mL in 20 mM TPBS), and the mixture was clotted by adding 50 μL of thrombin (50 NIH units/mL). Various amounts of fucoidan or its derivative dissolved in 150 μL of 20 mM TPBS were overlaid on the clots, and then the clot lysis time was determined at 37°. The method for determining the lysis of purified fibrin clots was the same as that described above, except that a plasminogen-rich human fibrinogen solution (8 mg/mL fibrinogen and 0.8 CU/mL Glu-plasminogen in 20 mM TPBS) was substituted for human plasma.

Determination of fibrin polymer formation. In polystyrene microcuvettes, 700 μL of a plasminogen-free fibrinogen solution (3.8 mg/mL in 20 mM TPBS) was mixed with various amounts of fucoidan or its derivative dissolved in 200 μL of 20 mM TPBS. After 50 μL of a thrombin solution (4 NIH units/mL in 20 mM TPBS) was added to the mixture, the A_{450} was measured continuously at 25° for 60 min. The initial rate of fibrin polymer formation was determined from the A_{450} during clotting, using the linear portion of each absorbance profile.

Protein determination. Protein concentration was determined by the method of Lowry *et al.* [13], using bovine serum albumin as the reference.

Animal experiments. Wistar male rats (5 weeks old, 110–120 g) were obtained from the Animal Center of Fukuoka University. The animals were divided into three groups (A and B, 5 rats per group; C, 4 rats per group) and kept under control conditions (temperature, 22 ± 2°; humidity, 50 ± 10%). The rats were maintained for 10 weeks on water and the following diet *ad lib.*: butter, 38%; cholesterol, 5%; sodium cholate, 2%; casein, 10%; cellulose, 10%; sucrose, 5%; vitamin mixture, 2%; standard laboratory chow, 28%. The B and C group rats received a weekly intravenous injection of highly sulfated fucoidan at the dosage of 1 and 2 mg/kg of body weight, respectively. The A group rats were injected with vehicle (saline) alone. Thrombosis in the hepatic veins was induced in each animal by intravenous injection of 0.3 mg/kg of body weight of endotoxin (*S. typhosa* lipopolysaccharide) 2 hr after the last fucoidan injection. They were deprived of the diet, but not water, for 17 hr before the endotoxin injection and killed with chloroform 24 hr after the endotoxin injection. The liver was weighed before and after removal of the red infarcted parts. Severity of thrombosis was quantitatively expressed in terms of percentage in grams of infarcted liver.

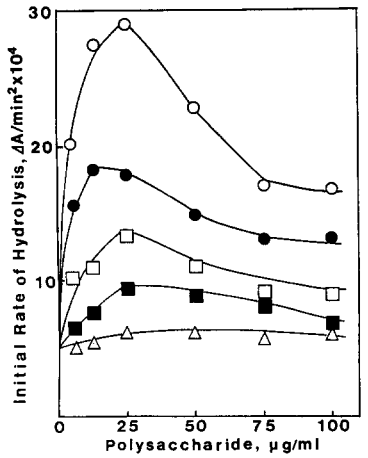

Fig. 1. Effects of native and chemically modified fucoidans on t-PA-catalyzed activation of Lys-plasminogen. The initial rate of Lys-plasminogen activation was measured as described in Materials and Methods. Symbol key: (□) native fucoidan; (○) highly sulfated fucoidan; (●) mildly sulfated fucoidan; (△) desulfated fucoidan; and (■) hepatin as a reference. Each point is the mean of duplicate experiments.

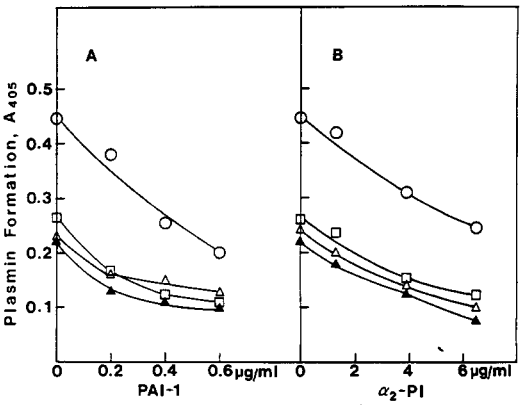

Fig. 2. Effects of native and chemically modified fucoidans on PAI-1 inhibition of plasmin generation (A) and on α_2-PI inhibition of plasmin activity (B). Each assay was performed in the presence or absence of 25 μg/mL fucoidan or its derivative as described in Materials and Methods. Activities obtained by highly sulfated fucoidan (○), native fucoidan (□), desulfated fucoidan (△), and without fucoidan (▲) are presented.

Other experimental details were as described previously by Renaud and Godu [14].

RESULTS

Chemical modification of fucoidan. By measuring sulfate concentration, as described in Materials and Methods, highly and mildly O-sulfated fucoidans were found to be 2.0 and 1.3 times more sulfated than native fucoidan, respectively. On the other hand, desulfated fucoidan contained only 1/6 of the sulfate group of native fucoidan.

Effects of native and chemically modified fucoidans on t-PA-catalyzed activation of plasminogen. Since native (Glu-) plasminogen was converted rapidly to the Lys-form by the plasmin formed during the assay, we chose Lys-plasminogen for determination of the effects of fucoidans on the plasminogen activation (Fig. 1). Among the fucoidans tested, highly sulfated fucoidan was the most effective stimulator. The highest stimulation (5.8-fold higher than without fucoidan) was obtained with 25 μg/mL. Mildly sulfated fucoidan and native fucoidan stimulated the plasminogen activation 3.6- and 2.6-fold, respectively, with 25 μg/mL concentrations. On the other hand, desulfated fucoidan showed no effect of stimulation. Heparin, which was used as a reference stimulator, showed only 1.9-fold stimulation with the same concentration. While amidolytic activities of t-PA and plasmin were not affected by 0–30 μg/mL fucoidan or its derivative, they were inhibited 20–25% by the addition of 50 μg/mL highly sulfated fucoidan (data not shown). The K_m values for the activation of Lys-plasminogen by t-PA in the absence and the presence of native fucoidan (15 μg/mL) were 10 and 9 μM, respectively, while the k_{cat} values were 0.035 and 0.095 sec^{-1}, respectively (data not shown).

Effects of native and chemically modified fucoidans on PAI-1 inhibition of plasmin generation and on α_2-PI inhibition of plasmin. The effects of fucoidan and its derivatives on PAI-1 inhibition of t-PA-catalyzed native (Glu-) plasminogen activation and on α_2-PI inhibition of plasmin were examined by using S-2251 as substrate. As shown in Fig. 2, the degree of inhibition by PAI-1 (panel A) and α_2-PI (panel B) did not change significantly in the presence of 25 μg/mL fucoidans. These results indicate that highly sulfated fucoidan exerts its stimulatory effect even in the presence of high concentrations of inhibitors.

Fibrinolysis by t-PA in the presence of highly sulfated fucoidan. Figure 3 shows the effect of various concentrations of highly sulfated fucoidan on human plasma clot lysis by t-PA. The sulfated fucoidan shortened the lysis time of plasma clot in a concentration-dependent manner from 0 to 26 μg/mL, and its effectiveness reached a maximum at 26 μg/mL. The maximal fibrinolytic activity of t-PA was 2.8 times over control in the presence of sulfated fucoidan, as judged from the calibration curve of t-PA concentration-dependent lysis time (data not shown). When more than 26 μg/mL of the fucoidan derivative was added, the lysis time began to be increased. Unlike plasma clot lysis, the sulfated fucoidan had no significant effect on the purified fibrin clot lysis by t-PA, as also shown in Fig. 3.

Fibrin polymer formation in the presence of fucoidan or its derivatives. Low concentrations of highly sulfated fucoidan (0.75 to 1.5 μg/mL) suppressed the fibrin polymerization rate by thrombin

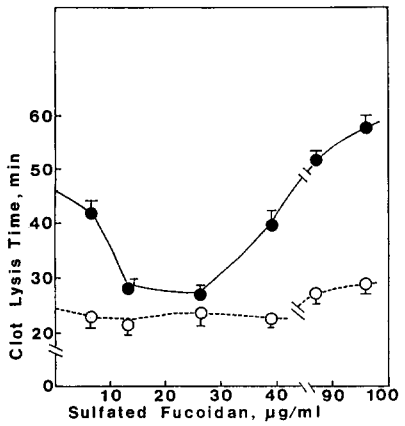

Fig. 3. Effect of highly sulfated fucoidan on human blood plasma (●) or purified fibrin (○) clot lysis caused by t-PA. Experimental details are described in Materials and Methods. Each point is the mean ± SD of four experiments.

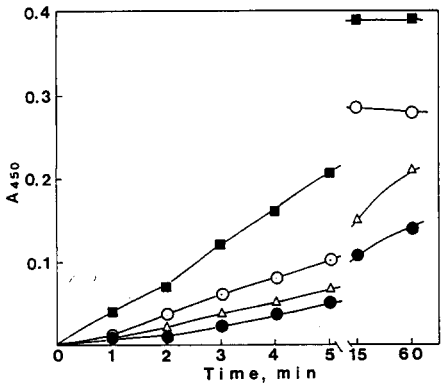

Fig. 4. Effects of hyaluronic acid and highly sulfated fucoidan on time-dependent fibrin polymer formation. Fibrinogen was incubated with thrombin in the absence (○) or in the presence of the following polysaccharides: 500 μg/mL hyaluronic acid (■), 0.75 μg/mL highly sulfated fucoidan (●) or 500 μg/mL hyaluronic acid plus 0.75 μg/mL highly sulfated fucoidan (△). Other experimental details are described in Materials and Methods. Each point is the mean of duplicate experiments.

to 13% of control. Native fucoidan at concentrations of 1.5 to 6 μg/mL also decreased the rate of fibrin polymer formation to 30% of control. At higher concentrations, the fucoidans had a tendency to aggregate fibrin(ogen) irreversibly, suggesting that they could bind fibrin(ogen) tightly. Desulfated fucoidan as well as heparin did not affect the fibrin polymer formation, although high concentration of heparin increased the rate of fibrin polymerization up to 2.1-fold (these data are not shown). As shown in Fig. 4, the fibrin polymer formation was stimulated by hyaluronic acid, as reported by LeBoeuf *et al.* [15]. On the other hand, highly sulfated fucoidan suppressed the polymerization to 50% of control. Moreover, hyaluronic acid stimulation was reduced markedly by the fucoidan derivative. These results show that native and highly sulfated fucoidans alter the kinetics of fibrin polymerization, and that the efficacy depends on the sulfation degree of the polysaccharide.

Effect of highly sulfated fucoidan on endotoxin-induced hepatic vein thrombosis in hyperlipemic rats. To examine the effect of highly sulfated fucoidan on the prevention of coagulation in an animal model, we created hepatic vein thrombosis model rats as described by Renaud and Godu [14]. Serum cholesterol levels, after feeding a butter-rich diet for 10 weeks, were 5-fold higher than those in the rats fed a laboratory chow. Thrombosis was induced in the model animals by endotoxin as described in Materials and Methods. As shown in Table 1, 100% of the animals developed thrombosis with a severity of 14 ± 5.5% (group A). However, the animals treated weekly with 1 mg/kg of highly sulfated fucoidan had thrombosis in only 20% of the animals with 0.4 ± 0.4% severity (group B). The rats injected with 2 mg/kg of the fucoidan derivative showed no hepatic vein thrombosis at all (group C).

DISCUSSION

We have shown that fucoidan and its sulfated derivatives stimulated t-PA-catalyzed Glu- or Lys-plasminogen activation according to the degree of sulfation (Figs. 1 and 2). When calculated on a molar basis, the abilities of native and highly sulfated fucoidans were 9.4 and 20.8 times that of heparin, respectively. Unlike heparin, the fucoidan derivative also promoted the generation of plasmin even in the presence of high concentrations of PAI-1 (Fig. 2). In addition, the sulfated fucoidan was an effective stimulator of the blood plasma clot lysis by t-PA (Fig. 3). The precise mechanism by which the sulfated fucoidan stimulates t-PA-catalyzed plasminogen activation and plasma clot lysis is not known. However, since t-PA and Lys-plasminogen bound to a fucoidan–Sepharose column (data not shown), fucoidan and its sulfated derivatives, like heparin [16] or fibrin [17], may serve as a "template" on which both molecules align in a conformation suitable for the plasminogen activation by t-PA. Under the situation, the active site of the generated plasmin may become relatively inaccessible to α_2-PI. Also, the augmentation of plasma clot lysis by sulfated fucoidan may be due to a predominant protective effect of the derivative on the inhibition of plasmin by α_2-PI rather than prevention of attachment of t-PA and plasminogen to the fibrin surface that promotes the plasmin generation.

We found that native and sulfated fucoidans decreased the polymerization rate of fibrin. This observation is in contrast to the fact that other polysaccharides such as hyaluronic acid, chondroitin sulfate and heparin have been reported to promote clot formation [15]. However, the effect of fucoidan was limited only at a low concentration range. At higher concentrations, fucoidan derivatives appeared to bind tightly to fibrin(ogen). We also found that highly sulfated fucoidan strongly inhibited the

Table 1. Effect of highly sulfated fucoidan on endotoxin-induced hepatic vein thrombosis in hyperlipemic rats

Group	Fucoidan treatment (mg/kg/week)	No. of rats	Severity of thrombosis (%)		P*
			Individual values	Mean ± SEM	
A	None	5	2.5, 3.7, 14.6, 20.0, 32.3	14.6 ± 5.5	
B	1.0	5	1.8, 0, 0, 0, 0	0.4 ± 0.4	< 0.025
C	2.0	4	0, 0, 0, 0	0	< 0.025

* Statistical significance of differences compared to the A group (Student's two-tailed *t*-test).

hyaluronic acid enhancement of fibrin polymer formation (Fig. 4). These observations suggest that the fucoidan derivative binds to fibrin(ogen) at the $A\alpha$254–380 region, which has been suggested as a hyaluronic acid binding site [18], or at the γ 373–410 region, which has been suggested as the polymerization site [19].

Plasminogen activator therapy for acute myocardial infarction has become standard medical practice. However, bleeding complications limit the use of available plasminogen activators. In addition, the prevention of reocclusion also remains an unsettled problem, since heparin cannot prevent coronary reocclusion in patients treated with t-PA [2, 3]. The limited efficacy of heparin may be due to a diminished inactivation of thrombin by heparin–AT-III on the clot surface [4, 5]. As described above, unlike heparin, fucoidan and its sulfated derivative can increase the fibrinolytic potency of t-PA in plasma and prevent clot formation by interacting with fibrin(ogen). Patients with disseminated intravascular coagulation syndrome (DIC) are reported to have greatly elevated levels of circulating hyaluronic acid that could bind to fibrinogen and enhance clot formation [20]. Therefore, the ability of highly sulfated fucoidan to reduce the hyaluronic acid-mediated enhancement of clot formation may prevent endotoxin (a DIC-causing factor)-induced thrombosis *in vivo*. We found that weekly administration of the sulfated fucoidan to hyperlipemic rats resulted in a significant diminution of the venous thrombi (Table 1). No firm conclusions can be drawn, at present, regarding the biochemical effects of fucoidan observed *in vitro* and the effects observed *in vivo*. However, a preliminary experiment with heparin (2 mg/kg) and hyperlipemic rats indicated that heparin was a weak effector on the prevention of endotoxin-induced thrombosis (the severities of thrombosis in three rats were 7.4, 1.5 and 0%). The comparative *in vivo* study with heparin and the sulfated fucoidan, and the investigation of the biochemical connection between *in vitro* and *in vivo* anticoagulant activities of the fucoidan are currently in progress.

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