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Roles of galactose and sulfate residues in sulfatides for their antagonistic functions in the blood coagulation system

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We previously reported that the sulfatide (galactosylceramide I^3-sulfate) may have contradictory functions, namely both coagulant and anticoagulant roles in vivo: sulfatide induced giant thrombi formation when injected into rats with vein ligation, whereas no thrombi were formed when sulfatide was injected into rats without vein ligation. Rather it prolonged bleeding time. To investigate the structural features of sulfatide for both functions, a synthetic sulfatide (galactosylceramide I^6-sulfate) which does not occur naturally, cholesterol 3-sulfate and ganglioside GM4 were examined together with naturally occurring sulfatide. Both sulfatides and cholesterol 3-sulfate induced giant thrombi in the rats with vein ligation within ten minutes of injection, although cholesterol 3-sulfate exhibited weaker coagulant activity than the sulfatides. On the contrary, both sulfatides significantly prolonged bleeding time but cholesterol 3-sulfate barely prolonged it when injected without vein ligation. GM4 exhibited neither coagulant nor anticoagulant activity. These results suggested that sulfate moiety in the sulfatides is essential for coagulant activity and that galactose residue enhances the activity, whereas both galactose and sulfate residues seem to be important for anticoagulant activity. This is because the sulfatides possess both residues but GM4 possesses galactose without sulfate and cholesterol 3-sulfate possesses sulfate without galactose. We previously reported that the possible mechanism of anticoagulation by sulfatide was due to its binding to fibrinogen, thereby inhibiting the conversion to fibrin. In this paper we reveal that both sulfatides inhibited thrombin activity independent of heparin cofactor II, thus providing evidence of another anticoagulation mechanism for the sulfatides.

Keywords: sulfatides, cholesterol 3-sulfate, blood coagulation, anticoagulation

Abbreviations: DVT, deep vein thrombosis; HCII, heparin cofactor II, PBS, phosphate buffered saline

Introduction

It has long been argued as to whether a sulfatide stimulates blood coagulation or inhibits it. In 1961, Wago reported prolongation of plasma clotting time in rabbits after administration of sulfatide [1]. In 1980, sulfatide was reported for the first time to activate blood coagulation factor XII (Hageman factor) *in vitro* [2]. Since then, sulfatide has been believed to be one important factor in the initiation of the intrinsic coagulation pathway, although the physiological importance of the intrinsic pathway starting from factor XII still remains unclear [3].

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Previouly, we have found that sulfatide prolongs clotting and bleeding times when administered to untreated animals, and have also shown its occurrence in many normal animal sera including human sera [4–7]. In order to investigate the role of sulfatide in thrombotic diseases, we examined its *in vivo* effects on a deep vein thrombosis model in rats, and found an unexpectedly marked enhancement of thrombogenesis 3 hours after sulfatide was injected into rats with vein ligation [8]. These observations suggest that sulfatide may work antagonistically in the blood coagulation system depending on the physical condition prevailing at that time. In this report, in order to investigate the structural features of the sulfatide for opposing functions, we compared coagulant and/or anticoagulant activities of four lipids related to sulfatide. These are two sulfatides: a naturally occurring one,

sulfatide-3S (galactosylceramide I^3-sulfate) and sulfatide-6S (galactosylceramide I^6-sulfate) together with ganglioside GM4 (I^3-α-*N*-acetylneuraminosylgalactosylceramide) and cholesterol 3-sulfate (5-cholesten-3β-ol sulfate).

Materials and methods

Materials

Male Sprague-Dawley rats (6.5–7.5 weeks of age) were obtained from Charles River Japan, Inc. (Atsugi, Japan). Sulfatide-3S and dermatan sulfate were prepared from pig spinal cord [6] and bovine intestinal mucosa [10], respectively. Sulfatide-6S was synthesized from galactosylceramide by sulfation with chlorosulfonic acid [9]. Cholesterol 3-sulfate and human thrombin were from Sigma (St. Louis, MO). GM4 was from Wako (Osaka, Japan) and the synthetic substrate for thrombin, S-2238 (Phe-Pip-Arg-pNA), was from Chromogenix AB (Mölndal, Sweden). Human plasma (normal and factor XII-deficient) were from Kokusai Shiyaku (Kobe, Japan). Thromboplastin ($CaCl_2$-free) from rabbit brain, used as tissue factor in this experiment, was from Böehringer Mannheim (Mannheim, Germany). Human heparin cofactor II (HCII) was from Calbiochem (La Jolla, CA).

Preparation of rat deep vein thrombosis (DVT) model

The rat thrombosis model was prepared as previously described [11]. Under anesthesia, the inferior vena cava was ligated just below the branch of the left renal vein with surgical silk thread. Lipids were suspended in phosphate buffered saline (PBS) with the aid of sonication as described [8]. These lipids (1 or 10 mg/kg) or the control vehicle were administered to rats as a bolus shot through the tail vein exactly 1 minute prior to the ligation. Rats in all groups were laparotomized 10 minutes after ligation and veins were incised to extirpate thrombi, and their weights were measured.

Measurement of onset time for plasma coagulation by turbidmetry

One volume of plasma was mixed gently with an equal volume of PBS (PBS-plasma). One hundred microliters of PBS-plasma containing lipids were added to wells of a 96-well microtiter plastic plate. The plate was installed in a microtiter reader (Wellreader SK603, Seikagaku Corp. Tokyo, Japan). Either $CaCl_2$ or $CaCl_2$ with "tissue factor" was added to each well to start coagulation. The increased absorbance due to fibrin formation was monitored at 405 nm every 15 seconds. The onset time of fibrin formation was defined as the first time of three sequential recordings at which the absorbance value in each well was elevated more than 0.02 from the base line [8].

Bleeding time test

Bleeding time tests were performed according to the transection model described by Dejana et al. [12]. Ten minutes after injection of lipids to rats through tail veins, the tips of the tails were cut transversely and the tails placed immediately in PBS warmed at 37°C. Bleeding time was defined as the time after which bleeding had completely stopped for more than 30 seconds.

Measurement of thrombin activity on a synthetic peptide

Fifty microliters of test samples, 25 µl of thrombin (0.25 mU/µl) and 25 µl of HCII (0.04 µg/µl) were mixed in a well of a 96-well microtiter plastic plate. Immediately after adding 50 µl of synthetic substrate S-2238 (2 mM) to a well, the plate was installed in a microtiter reader (Wellreader SK603, Seikagaku Corp. Tokyo, Japan). The increased absorbance was monitored every 15 seconds at 405 nm with 492 nm being used as a reference wavelength. The catalytic rate of thrombin activity was calculated as $\Delta OD/\Delta time$. Residual thrombin activity was defined by the equation.

$$\text{Residual thrombin activity} = (A - B/C - B) \times 100\ (\%)$$

where A is a value in the presence of thrombin, HCII and test samples; B is a value in the absence of thrombin, HCII and sulfated conjugates; C is a value in the presence of thrombin and HCII but no test samples.

Results

Coagulation effects of lipids in *in vivo*: effects on DVT model

The effects of sulfatides, cholesterol 3-sulfate and GM4 on thrombogenesis *in vivo* were examined using the DVT model (Figure 1). All sulfated lipids markedly induced thrombi in rats with vein ligation whereas non sulfated lipid GM4 did not. The mode of induction of thrombi by the sulfatides and cholesterol 3-sulfate differed from one another. The sulfatides showed a bell-shape dependence; sulfatides made heaviest thrombi at doses of 1 mg/kg whereas cholesterol 3-sulfate induced thrombi in a dose-dependent manner (data not shown for sulfatide 6S). We previously reported no induction of thrombogenesis by sulfatide-3S if it were simply injected into rats without vein ligation. This phenomenon was also observed with sulfatide-6S and cholesterol 3-sulfate (data not shown).

Effects of lipids on plasma coagulation times initiated by $CaCl_2$

The coagulation onset times initiated by $CaCl_2$ were measured using rat and human plasma and the patterns were similar (Figure 2). Sulfatide-3S and sulfatide-6S exhibited similar coagulant activities, with the latter showing slightly stronger activity. For example in rat serum without lipids, the onset time was 7.15 minutes whereas in the presence of sulfatide-3S and sulfatide-6S at 93.75 µg/ml, onset times were at 2.18 and

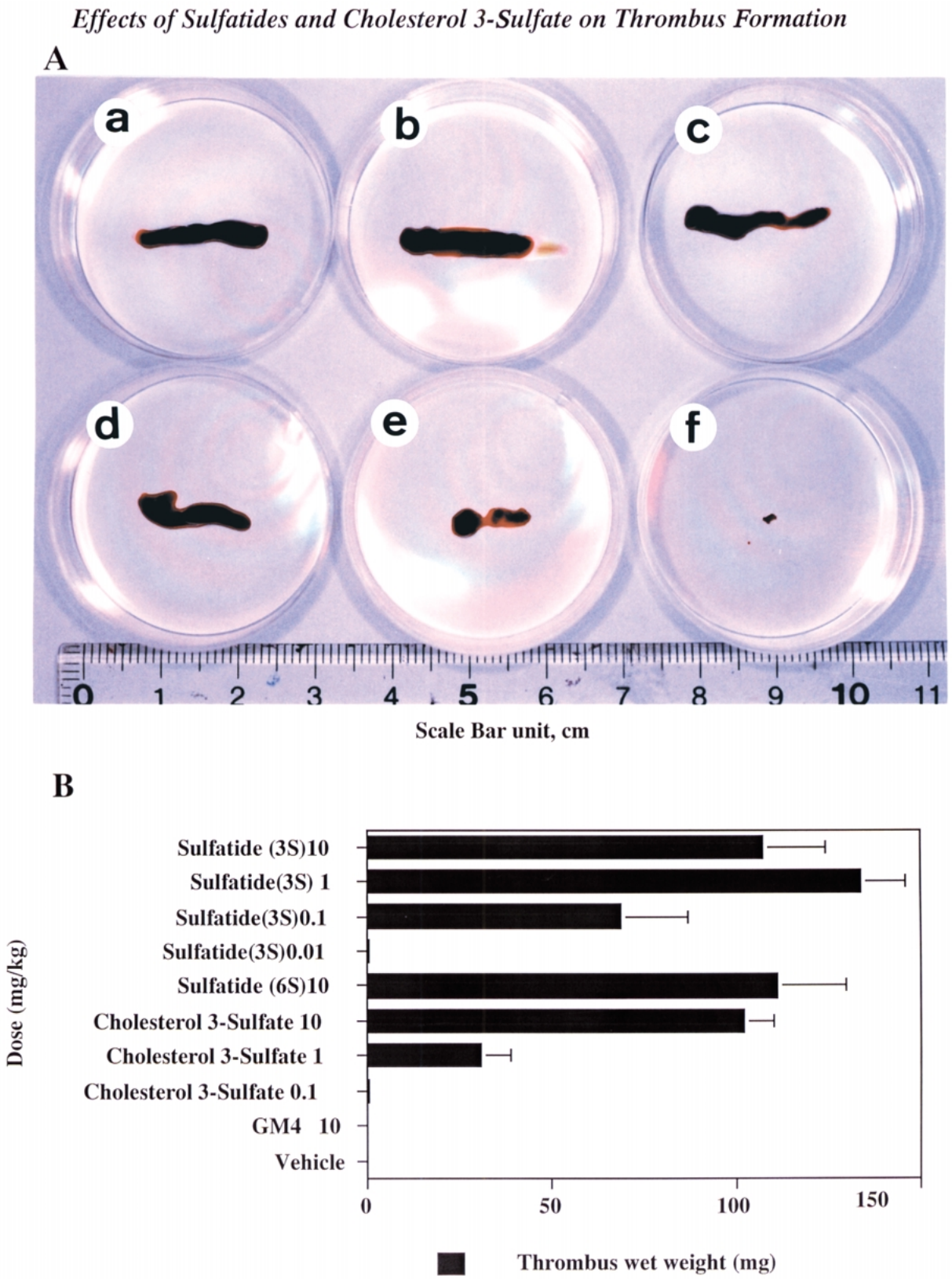

Figure 1. The effect of lipids on thrombogenesis in a rat deep vein thrombosis model. A. a–f, Thrombi induced by 10, 1, 0.1 mg/kg of sulfatide-3S and 10, 1, 0.1 mg/kg of cholesterol 3-sulfate, respectively. (Scale, cm) B. The weights of thrombi 10 minutes after adminstration of the lipids to rats with vein ligation. (n = 3, mean value ± SE).

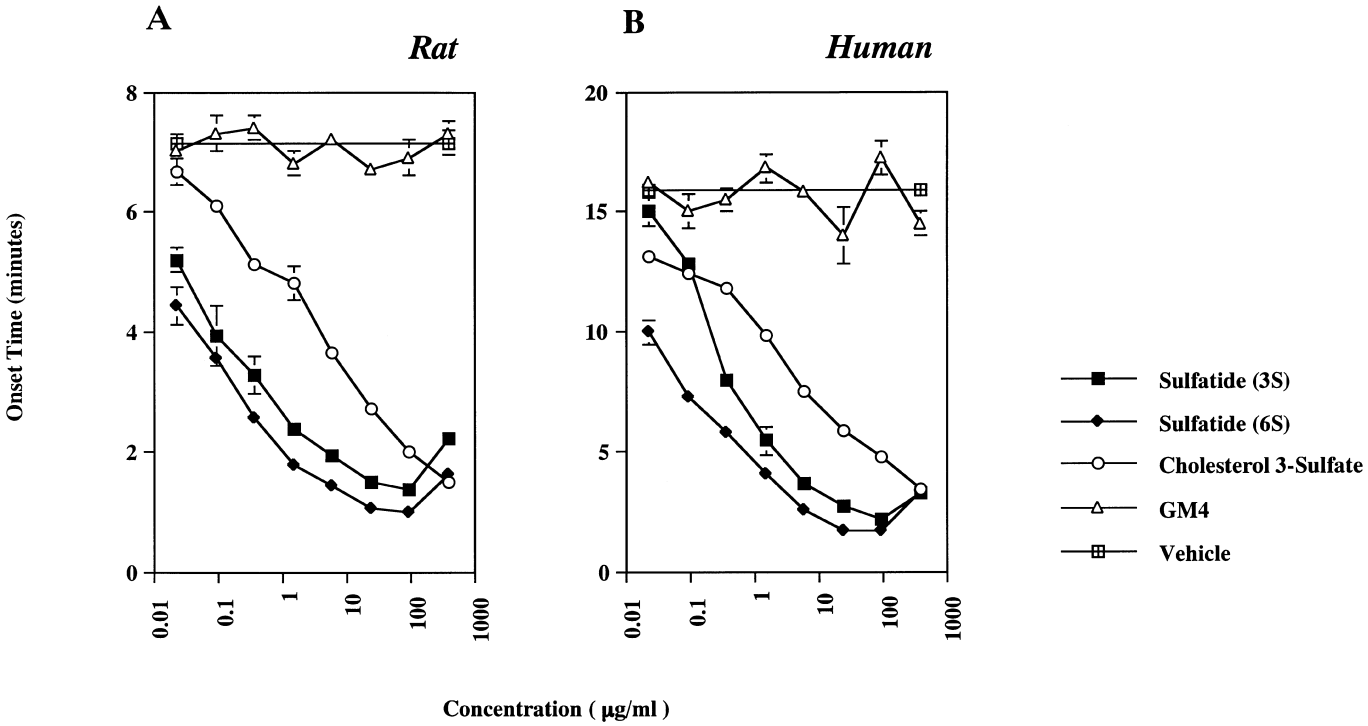

Figure 2. The effects of lipids on coagulation onset time initiated by $CaCl_2$ in rat (A) and human plasma (B). Patterns were similar. Sulfatide-3S and sulfatide-6S showed basically similar coagulant activities, although the latter had slightly stronger activity. Cholesterol 3-sulfate exhibited weaker coagulant activity than either of the sulfatides. GM4 exhibited no activity. (n = 3, mean value ± SE).

1.68 minutes respectively. Both sulfatides shortened the time in a dose-dependent manner between 0.023–93.75 μg/ml but at 375 μg/ml, the effect was weaker, both sulfatides displaying bell-shaped behavior. Cholesterol 3-sulfate exhibited weaker coagulant activity than both sulfatides but its effect was a dose-dependent one. In contrast, GM4 exhibited no activity. These results are consistent with those shown *in vivo* in the rat DVT model. When these sulfated lipids were examined using factor XII-deficient plasma in this system, no coagulation was observed (data not shown).

Anti-coagulation effects of sulfated lipids in *in vivo*: effects on bleeding time (bleeding time test)

The effect on bleeding time in rats was measured without vein ligation, 10 minutes after injection of 10 mg/kg of sulfated lipids. The conditions of dose and time were found to be sufficient for all lipids to generate thrombi in the DVT assay (Figure 1). Without lipid, the bleeding time of rats was approximately 12 minutes and was not significantly prolonged by cholesterol 3-sulfate. On the contrary, in the presence of either sulfatide, bleeding time was markedly prolonged (more than 30 minutes).

Effects of lipids on thrombin activity on synthetic substrate

We examined the effects of lipids on thrombin activity in the presence and absence of heparin cofactor II and compared to those of GM4 and dermatan sulfate. As shown in Figure 3, both sulfatides (but not cholesterol 3-sulfate and GM4) inhibited thrombin activity in a dose-dependent manner to a similar extent. This result was consistent with the *in vivo* results shown in bleeding time test. Furthermore this inhibitory activity was unrelated to HC II, well known as an anti-thrombin protein whose action is enhanced by sulfated glycoconjugates such as heparin [13] dermatan sulfate [14], dextran sulfate [15] and fucosylated glycosaminoglycans [16,17].

Effects of sulfated lipids on factor XII-deficient plasma coagulation times initiated by tissue factor

We previously reported that sulfatide 3-S prolonged the plasma onset time of blood coagulation factor XII-deficient plasma [8]. In the present study, we compared sulfatide-3S, sulfatide-6S cholesterol 3-sulfate and GM4. Both sulfatides prolonged onset time of coagulation dose-dependently, in contrast to no effects of cholesterol 3-sulfate and GM4 (Figure 4). This result is also consistent with the *in vivo* data of bleeding time test.

Discussion

We previously reported antagonistic roles for sulfatide-3S in the blood coagulation system [7,8]. In summary the results were: i) Sulfatide-3S prolongs bleeding time when simply injected as a bolus shot to animals without any treatment

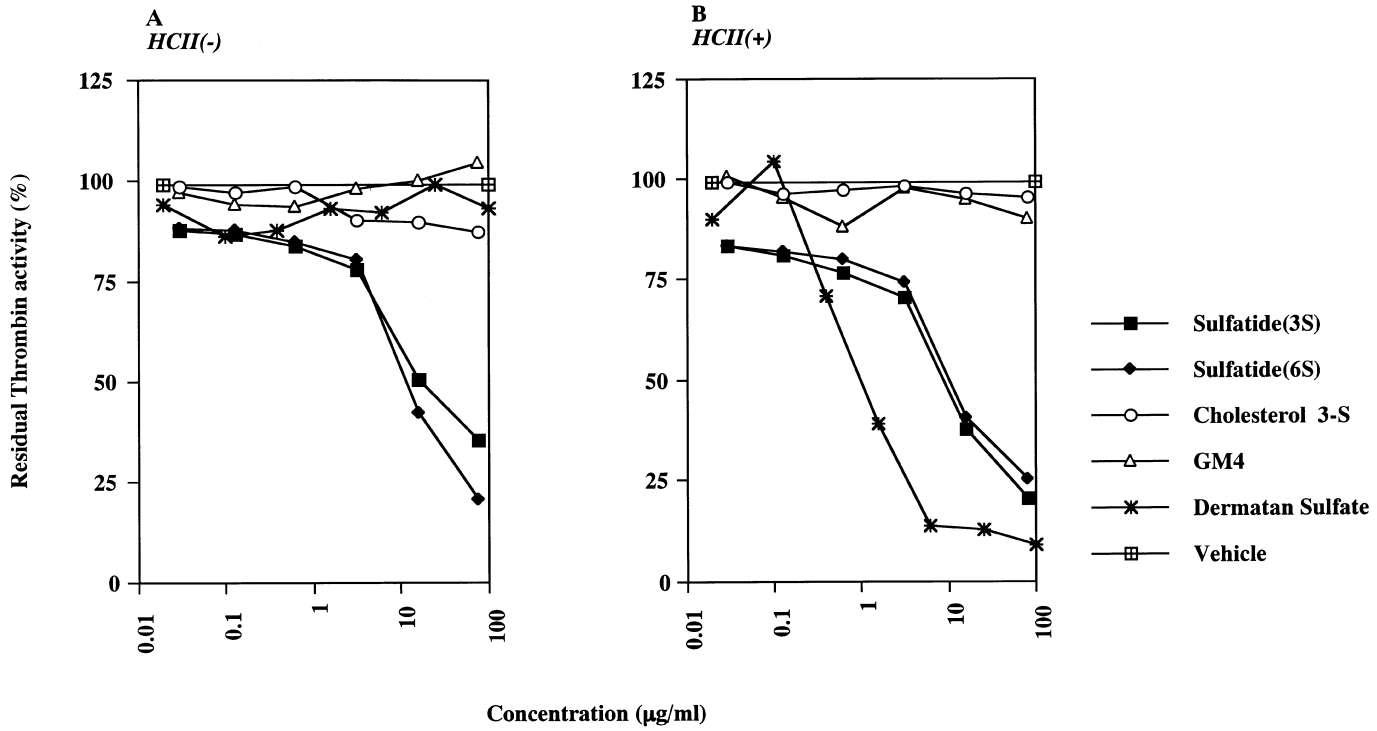

Figure 3. The effects of lipids on thrombin activity towards synthetic substrate. Both sulfatides (but not cholesterol 3-sulfate and GM4) inhibited thrombin activity dose-dependently in an almost identical manner regardless of the absence (A) or presence (B) of heparin cofactor II. Dermatan sulfate, by contrast, requires heparin cofactor II for inhibiting thrombin activity [13,14].

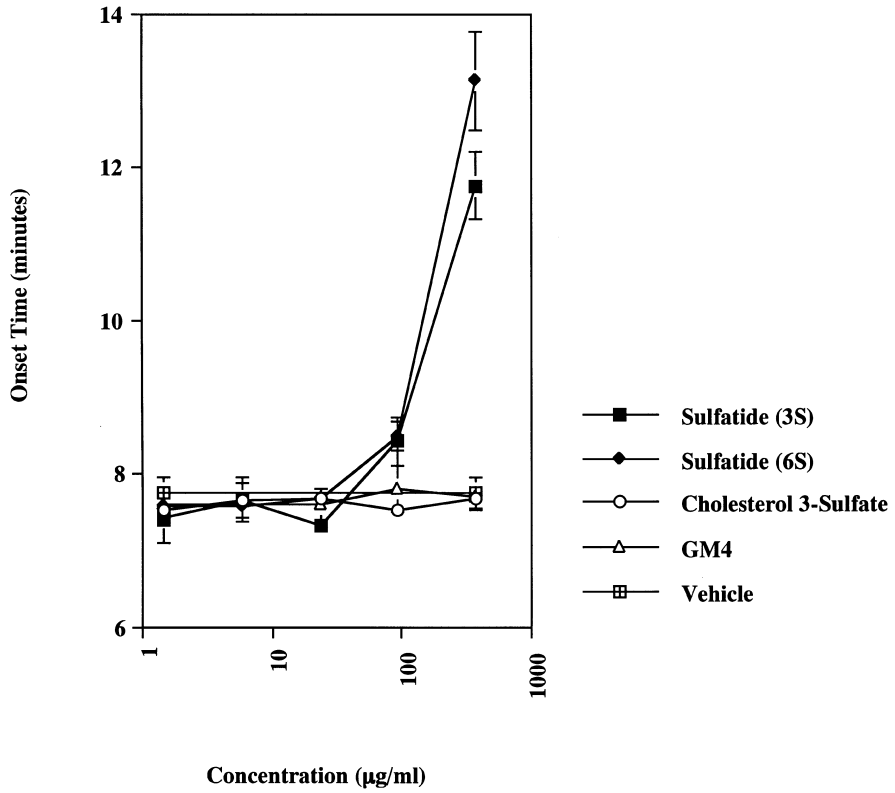

Figure 4. The effects of lipids on factor XII deficient plasma coagulation times initiated by tissue factor. Both sulfatides prolonged the time of onset of blood coagulation in a dose-dependent manner, whereas cholesterol 3-sulfate and GM4 did not.

[4,6,7] and this anticoagulant activity seemed due to interferences with conversion of fibrinogen to fibrin by binding to fibrinogen [6,7]. ii) Sulfatide-3S generated giant thrombi when injected as a bolus shot to animals with vein ligation [8].

In addition, we show in this report that for coagulant activity sulfate residue in the lipids is essentially important because both sulfatides and cholesterol 3-sulfate, but not GM4, exhibited coagulant activity not only *in vitro* but also *in vivo* and that galactose residue contributes to enhancement of this activity. It is also shown that for anticoagulant activity both the galactose and sulfate residues of the sulfatides are important because only sulfatides, but not cholesterol 3-sulfate lacking galactose residue and GM4 lacking sulfate residue, exhibited anticoagulant activity. With respect to the positioning of the sulfate moiety in galactose, sulfatide-6S exhibited slightly stronger coagulant activity than sulfatide-3S but both sulfatides exhibited almost equal anticoagulant activity. Further we found that both sulfatides inhibited thrombin without the involvement of heparin cofactor II. We have already reported on the absence of participation of antithrombin III in the anticoagulant activity of sulfatide [5]. Unlike sulfatides, glycosaminoglycans, which are other types of sulfated glycoconjugates, have a requirement for naturally occurring anticoagulant proteins: heparin exhibits anticoagulant activities via antithrombin III [18–20] and/or heparin cofactor II [13], and dermatan sulfate exhibit anticoagulant activities via heparin cofactor II [14,21].

Plasma coagulation experiments initiated by $CaCl_2$ indicate that in order to exhibit coagulant activity, both sulfatides and cholesterol 3-sulfate required blood coagulation factor XII. The activation of factor XII by these sulfated lipids on plastic surface was also reported by others [2,22,23]. Nevertheless, the role of factor XII has been considered to be non-physiological and nowadays, the pathway starting from tissue factor is believed to be important in blood coagulation systems [3]. However, our *in vivo* results from both coagulation and anticoagulation experiments seem consistent with the *in vitro* results for both antagonistic functions, suggesting that the pathway involving factor XII may operate *in vivo*, especially in pathological cases. Sulfatides induced giant thrombi within 10 minutes, suggesting that this factor or a related molecule may be interacting with sulfatide to initiate coagulation, and may exist independently of *de novo* protein synthesis in animal serum.

The pathogenesis of venous thrombosis is unclear but generally venous stasis with vessel wall damage has been considered necessary [24]. Endogeneous sulfatide occurs naturally in many animal sera and blood cells [4,25,26]. Since glycosphingolipids have been reported to exist in densely populated regions of the cell surface [27], endogenous sulfatide in such domains or the sulfatide shed from the cells [26] may induce thrombosis under venous stasis together with possible involvement of factor XII.

By contrast, in the absence of venous obstruction or stasis, sulfatide may exhibit anticoagulant activity, because bleeding times were significantly prolonged when sulfatide was injected into untreated rats. In addition to binding to fibrinogen, thereby inhibiting the conversion fibrinogen to fibrin [6,7], we demonstrated another mechanism whereby sulfatides directly inhibit thrombin activity without involvement of heparin cofactor II.

We have focused function of sulfatide mainly on plasma coagulation proteins [4–8]. However, many other components such as platelets, monocytes and vessel wall, all of which were reported to contain sulfatide [28–31], are involved in thrombotic diseases. Therefore to investigate sulfatide roles in cardiovascular diseases is the next important subject. Indeed we already reported abnormal accumulation of sulfatide in the atherosclerotic aorta of Watanabe hereditable hyperlipidemic rabbit [30]. Sulfatide occurs ubiquitously and binds many endogenous proteins specifically or non-specifically [31], sometimes prompting the question as to whether these phenomena triggered by sulfatide represent real physiological functions of sulfatide. Although these various functions of sulfatide sometimes seem to contradict each other as reported in this paper, such properties may fortuitously lead to the induction of diseases, emphasizing the importance of studying sulfatide function, especially in the medical field.

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