Glycoconjugate Journal 15, 915–922 (1998)

Sulfatide can markedly enhance thrombogenesis in rat deep vein thrombosis model

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Although sulfatide (galactosylceramide I[3]-sulfate) has been reported to activate blood coagulation factor XII (Hageman factor), it has been administered to animals without subsequent thrombus formation. We recently found that sulfatide binds to fibrinogen and thus disturbs fibrin formation *in vitro*, suggesting its possible role as an anticoagulant rather than as a coagulant. We therefore examined the *in vivo* effects of sulfatide on thrombogenesis by using a rat deep vein thrombosis model in which thrombus is induced by ligating the inferior vena cava. Sulfatide and gangliosides were each separately administered to rats 1 min before the vein ligation, and after 3 h, sulfatide but not gangliosides markedly ($P < .001$) enhanced the thrombogenesis. A kinetic turbidmetric assay of plasma coagulation initiated by $CaCl_2$ in the wells of a microtiter plate revealed that coagulation was also markedly accelerated in the presence of sulfatide but not gangliosides, the results of which seemed to be very consistent with those of the *in vivo* experiments. Because sulfatide could not induce thrombosis without vein ligation in rats, the enhancement of thrombogenesis by sulfatide in the *in vivo* model might require endothelial damage and/or venous congestion, both of which could be induced by vein ligation.

Keywords: **sulfatide, enhanced thrombogenesis by sulfatide *in vivo***

Abbreviations: **WHHL, Watanabe hereditable hyperlipidemic; DVT, deep vein thrombosis; PT, prothrombin time; PBS, phosphate buffered saline**

Introduction

In 1961, before the structural confirmation of sulfatide (galactosylceramide I[3]-sulfate) [1], Wago reported possible anticoagulant and anti-atherosclerotic activities of sulfatide [2]. He administered sulfatide orally or intravenously to rabbits, and observed not only a prolongation of plasma clotting time but also a decrease in the plasma cholesterol level with an improvement of atheromatous involvement in the aorta. About twenty years later, sulfatide was reported for the first time to be able to activate blood coagulation factor XII (Hageman factor) *in vitro* [3]. Since then, sulfatide has been believed to be one of the important factors in the initiation of the intrinsic coagulation pathway [4, 5], although the physiological importance of the intrinsic pathway itself still remains unclear [6, 7]. On the contrary, sulfatide has been administered to animals without subsequent thrombus formation [1, 8–10]. In 1987, we found for the first time the occurrence of sulfatide in Watanabe hereditable hyperlipidemic (WHHL) rabbit blood as a component of lipoprotein [11] and subsequently found its natural occurrence in sera from various species, including humans [9]. Furthermore, accumulation of sulfatide was observed in the atherosclerotic aorta of WHHL rabbit [12]. Recently, we found that sulfatide prolongs the plasma coagulation time and bleeding time, probably due to its binding to fibrinogen and subsequent disturbance of fibrin formation [10], suggesting that it may have anticoagulant activity *in vivo*. These data imply that sulfatide in plasma may have important roles in blood coagulation and atherosclerosis. In the present study, therefore, we examined its possible anticoagulant activity using a rat deep vein thrombosis (DVT) model in which thrombus is experimentally induced by ligating the inferior vena cava [13]. Sulfatide was administered to rats 1 min before the vein ligation to prevent thrombus formation. Unexpectedly, however, the sulfatide markedly enhanced the thrombogenesis in these rats. A kinetic turbidmetric assay of plasma coagulation was carried out in the wells of a microtiter plate. Coagulation was markedly accelerated in the presence of sulfatide, the result of which seemed to be very consistent with that of the *in vivo* experiment.

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Materials and methods

Materials

Male Sprague-Dawley rats, 6.5–7.5 weeks aged were obtained from Charles River Japan, Inc. (Atsugi, Japan). Sulfatide was prepared from pig spinal cord in our laboratory as described previously [10]. Ganglioside GM1 from bovine brain and GD3 from bovine milk were purchased from Sigma (St. Louis, Missouri) and from Wako (Osaka, Japan), respectively. Human plasma (normal and factor XII-deficient) and prothrombin time (PT) reagent composed of thromboplastin with $CaCl_2$ were purchased from Kokusai Shiyaku (Kobe, Japan). Thromboplastin ($CaCl_2$; free), used as tissue factor in this experiment, from rabbit brain was purchased from Boehringer Mannheim (Mannheim, Germany).

Preparation of an experimental thrombosis model

The rat thrombosis model was prepared as described [13]. After the rat was laparotomized under anesthesia with Nembutal^{RTM} (Abbott, North Chicago, Illinois), the inferior vena cava was ligated just below the branch of the left renal vein with surgical silk thread (diameter 0.48–0.56 mm). Sulfatide and gangliosides were suspended in phosphate buffered saline (PBS) with the aid of sonication at the concentration 1 or 10mg/ml. Although it is difficult to suspend these glycolipids (especially sulfatide) homogeneously in H_2O or physiological saline, they are relatively easily soluble as homogeneous micellar suspensions in PBS. These acidic glycolipids (1 or 10 mg/kg) were administered into rats as a bolus shot through the tail vein exactly 1 min prior to the ligation. Two control groups were prepared. In one group, PBS (vehicle) was injected the same way through the tail vein 1 min prior to ligating the inferior vena cava. In another group, sulfatide was injected through the tail vein without vein ligation. The rats in all groups were laparotomized under ether anesthesia 3 h after the ligation, and the veins were incised to extirpate thrombi, the weights of which were measured.

Kinetic analysis of plasma coagulation initiated by $CaCl_2$ using a 96-well microtiter plate

Rat and human plasma were used in this analysis, which was originally designed for Limulus test to determine bacterial endotoxins [14]. Rat plasma was prepared from the freshly drawn blood using 3.2% trisodium citrate as an anticoagulant. Commercially available human plasma was reconstructed according to the manufacturer's protocol. One volume of plasma was mixed gently with the same volume of PBS, which is called as PBS-plasma hereafter. One hundred–microliter aliquots of PBS-plasma containing various amounts of sulfatide were added to the wells of a 96-well microtiter plastic plate (polystyrene flat bottom, Corning 25801, Corning, New York). The plate was installed in a microplate reader (Wellreader SK601, Seikagaku Corp., Tokyo, Japan) and kept at 37°C during the analysis. Twenty-five μl of 0.02M $CaCl_2$ was added into each well to activate multiple plasma coagulant factors for starting the coagulation. The increase of absorbance by the increased turbidity due to fibrin formation was monitored at 405 nm and recorded every 15 s. The onset time of fibrin formation in this analysis was defined as the first time of three sequential records when the absorbance value in each well is continuously elevated more than 0.02 from the base line.

Kinetic analysis of plasma coagulation initiated by tissue factor using a 96-well microtiter plate

Commercially available human normal and factor XII-deficient plasma were used in this study. After 100 μl aliquots of PBS-plasma containing 0.2 μg/ml of tissue factor with or without various amounts of sulfatide were added to the wells, 25 μl aliquots of 0.02M $CaCl_2$ were added to the wells to start the coagulation. The increase of the absorbance and the onset time were monitored and recorded as described above.

PT tests

One hundred–microliter of rat PBS-plasma containing sulfatide was incubated for 1 min at 37°C. Coagulation was started by adding 200 μl of PT reagent, and the onset time of coagulation was determined with an automatic coagulometer (Amelung KC-10A, Baxter, Irvine, California), which can detect the change of plasma viscosity.

Results

Effect of sulfatide on experimental thrombosis in rats

We used a rat thrombosis model corresponding to human deep vein thrombosis [13]. This model has often been used for evaluation of the potential of anticoagulant agents whose efficacies are usually analyzed at 3 or 4 h after injection of the agents by comparing the weight of thrombi lessened by the agents with that by the vehicle [15]. When 10 mg/kg of sulfatide was simply injected into rats without vein ligation, as expected, the rats appeared to be normal and no thrombus was observed at all (Figure 1A). However, as shown in Figure 1, both doses of 10mg/kg and 1mg/kg remarkably enhanced thrombogenesis in the rats with vein ligation. The thrombi induced by injection of 1mg/kg of sulfatide were slightly but significantly ($P < 0.05$) larger than those induced by the injection of 10mg/kg of sulfatide. The other negatively charged glycolipids GM1

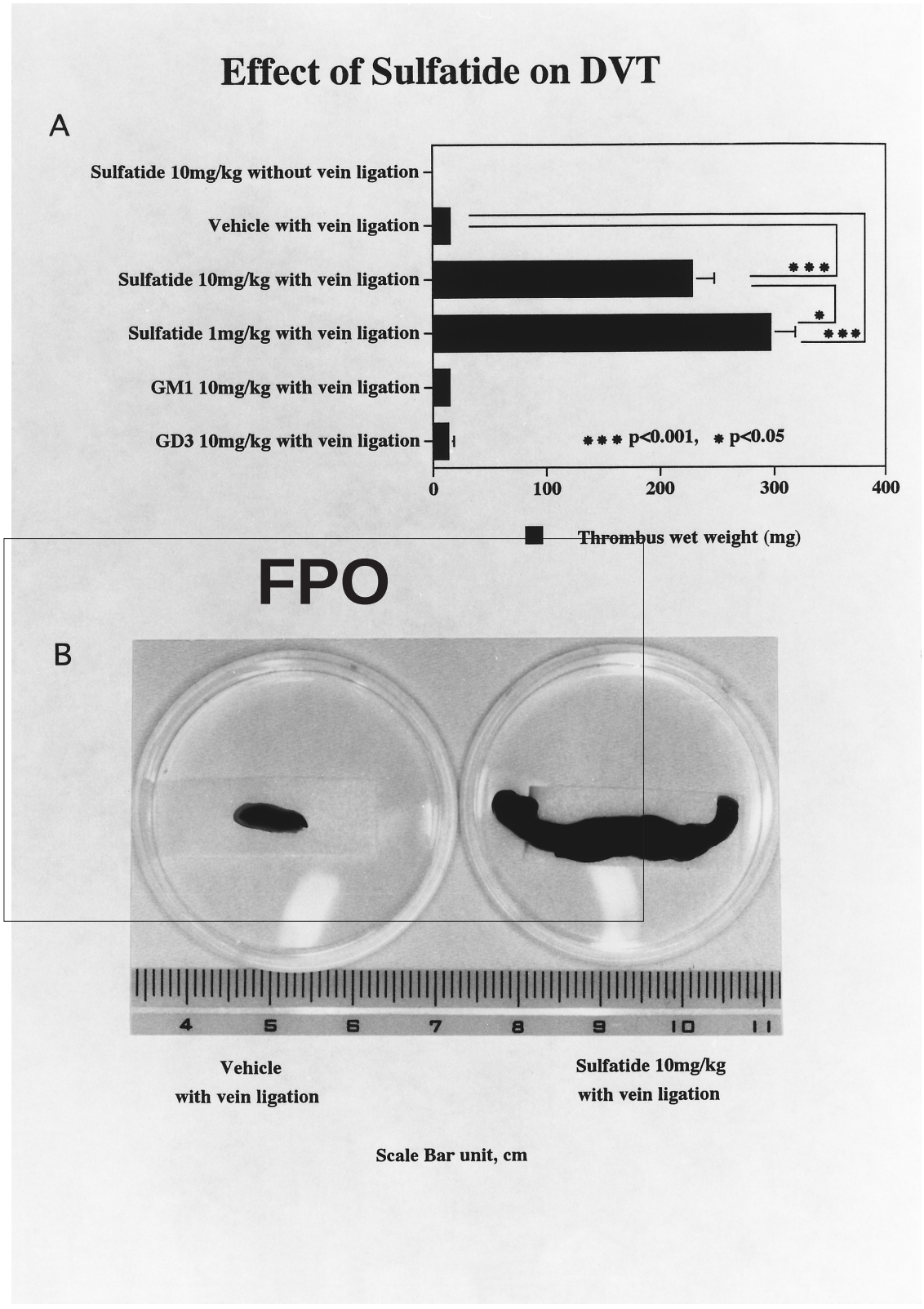

Figure 1. (A). The effect of sulfatide on thrombogenesis in a rat deep vein thrombosis model. The weights of thrombi 3 h after the adminstration of glycolipids are presented. The thrombi weights of rats administered sulfatide at 10mg/kg or 1 mg/kg with vein ligation were significantly ($P < 0.001$) increased compared with that of the vehicle-treated rats. The thrombi weight of the rats adminstered 10mg/kg of sulfatide were slightly but significantly ($P < 0.05$) decreased compared with those of the rats administered 1mg/kg sulfatide. (B). The left one is a thrombus from the control group administered the vehicle with vein ligation, and the right one is from a rat given 10 mg/kg of sulfatide with vein ligation (Scale, cm).

and GD3 did not enhance thrombogenesis in this rat model (Figure 1A).

Effect of sulfatide on plasma coagulation times observed on a 96-well microtiter plate

To evaluate the coagulant activity of animal plasma, we created a new method as described in Materials and Methods. As shown in Figure 2, rat PBS-plasma (control) slowly started to coagulate at 13.1 min after the addition of $CaCl_2$ to the wells. Sulfatide (23 μg/ml) apparently accelerated this process starting at 3.30 min, but this effect appeared to be abated at a high concentration (375μg/ml). Figure 3 shows the relationships between the plasma coagulation onset time and the glycolipid concentration in rat and human plasma. There are clear linear relationships between the concentrations of sulfatide (0.023-23 μg/ml) and the coagulation onset times when both parameters are plotted as logarithmic numbers [for rat plasma, $Y=-0.2148X+0.810736$, $r=0.999$; for human plasma, $Y=-0.2871X+0.8194$, $r=0.995$, $X=\log_{10}$(sulfatide μg/ml), $Y=\log_{10}$(min)]. In both types of plasma, at the concentrations of sulfatide between 0.09 and 23.44μg/ml, the plasma coagulation onset times were shortened dose-dependently, but at above 93.75μïml, this phenomena seemed to be reversed. On the other hand, the other negatively charged glycolipids GM1 and GD3 showed little, if any, activity. After the injection of glycolipids into rats, their concentrations in the blood can be calculated as 130μg/ml (corresponding to the 10mg/kg case) and 13μg/ml (1mg/kg), respectively, by assuming that the glycolipids are instantly and equally distributed in the blood and that the circulating blood volume in animals is 8% of its whole body weight [16]. As shown in Figure 3A, the onset times corresponding to these concentrations in the above-mentioned *in vitro* assay may be estimated to be 3.71 min (1mg/kg case) and 4.88 min (10mg/kg case), respectively, whereas the onset time without sulfatide was 13.1 min. Therefore, these concentrations of sulfatide in the blood may be enough to strongly accelerate blood coagulation. It may also be reasonable that 1mg/kg of sulfatide could more significantly enhance thrombogenesis than 10mg/kg of it *in vivo*. It should be noted that the *in vitro* result from rat plasma seemed similar to that from human plasma. In addition, the result from the *in vitro* assay seemed to be consistent with that from the *in vivo* assay found in rats. Taken together, these data suggest that thrombosis induced by sulfatide may also occur in humans. The kinetic analysis of plasma coagulation initiated by $CaCl_2$ indicates that the coagulation is induced through the activation of factor XII on a plastic surface [3]. We also confirmed this by use of factor XII-deficient plasma. The XII-deficient plasma-PBS did

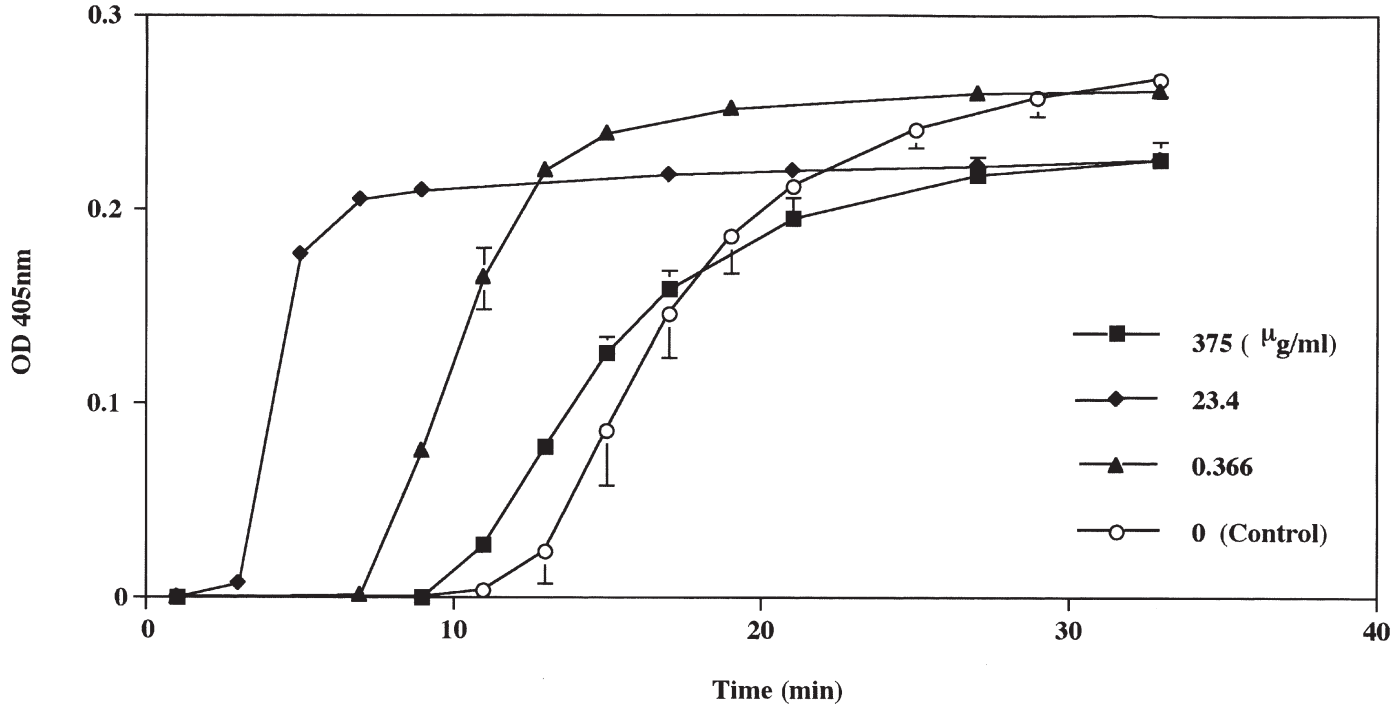

Figure 2. Effect of sulfatide on rat plasma coagulation time in microtiter plate wells. Aliquots of 100 μl of rat plasma-PBS were added to the wells of a 96-well microtiter plate, and coagulation was initiated by adding $CaCl_2$. Rat plasma–PBS slowly started to coagulate at about 12 min. Sulfatide (23.4 μg/ml) could apparently accelerated this process at about 3 min, and the accelerating activity was still observed at a dose of 0.37 μg/ml. By contrast, at a relatively high concentration (375μg/ml), the accelerating activity seemed to be abated. The symbols represent the means of triplicate experiments, and vertical bars represent SE.

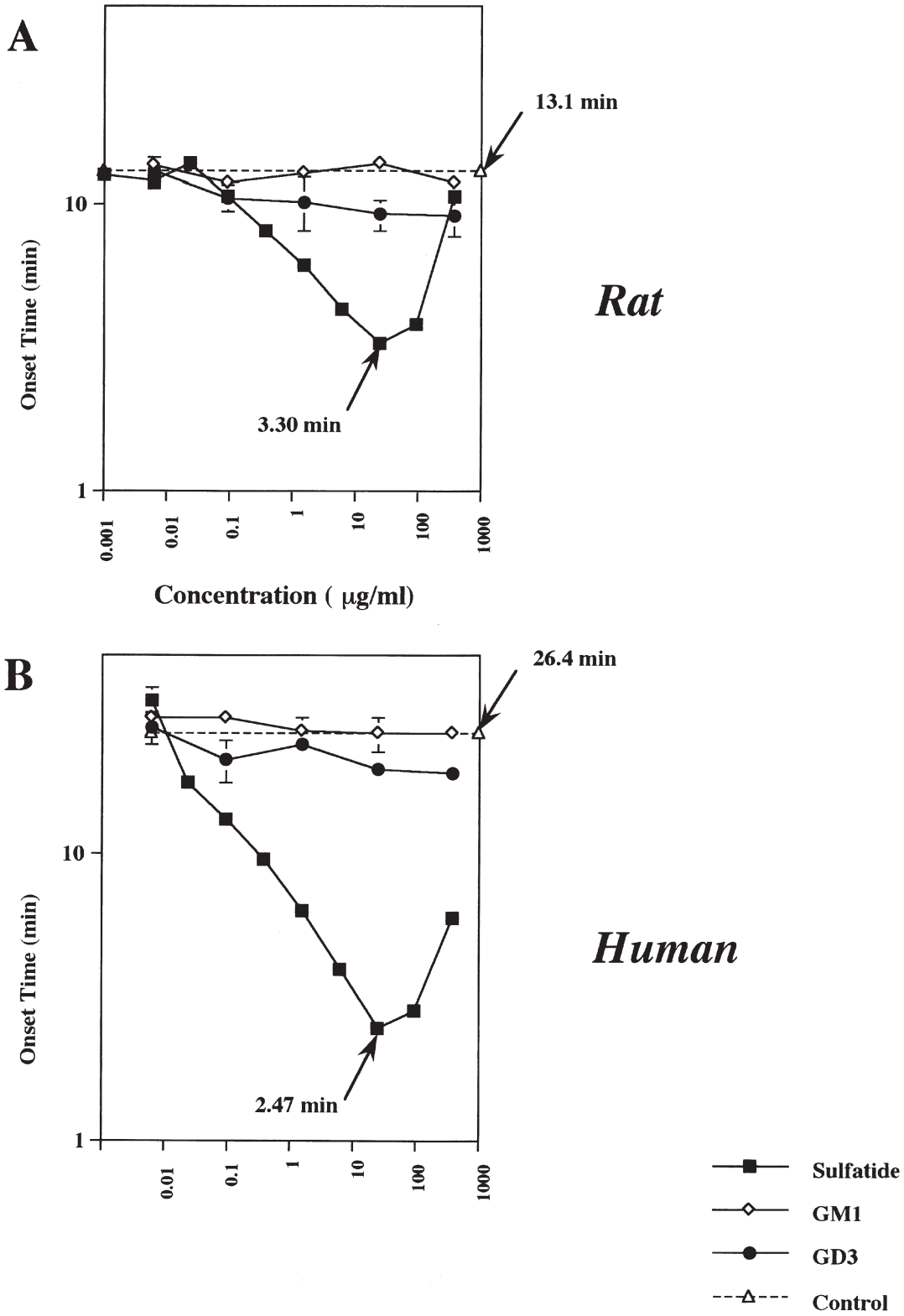

Figure 3. The relationships between sulfatide concentration [$\log_{10}$ (μg/ml)] and coagulation onset time [$\log_{10}$(min)] initiated by $CaCl_2$ in rat (A) and human plasma (B). Sulfatide could accelerate plasma coagulation at a dose as small as 0.089 μg/ml, and had a maximum effect at 23 μg/ml in both types of plasma. At more than 100μg/ml, however, this acceleration effect was weakened. The gangliosides GM1 and GD3 had no or little effect on either type of plasma. The symbols represent the means of triplicate experiments, and vertical bars represent SE.

not coagulate either in the presence or absence of any amount of sulfatide in the kinetic analysis (data not shown). Further, to see the effect of sulfatide on the blood coagulation system in the absence of factor XII, coagulation was initiated by the addition of $CaCl_2$ and tissue factor, the latter of which can induce fibrin formation regardless of the presence of factor XII [17]. As shown in Figure 4, in the deficient plasma, at more than 0.37 μg/ml of sulfatide, the onset time was prolonged dose-dependently, which was probably due to its binding ability to fibrinogen, disturbing fibrin formation as was reported [10]. On the other hand, in the case of normal plasma, the net reaction accompanied by both coagulant activities, example, one from factor XII activation and the other from tissue factor, was observed. In contrast, prolongation of the coagulation time was not observed at between 5.86 and 93.75 μg/ml of sulfatide, but the onset time was suddenly shortened with the increase of the concentration. Finally, at more than 93.75 μg/ml of sulfatide, this shortening was again abated as was found in the coagulation assay initiated by $CaCl_2$ without tissue factor. These observations suggest that sulfatide may still have anticoagulant activity under the inactivation of factor XII where the rats injected with sulfatide without vein ligation may coincide with the above case.

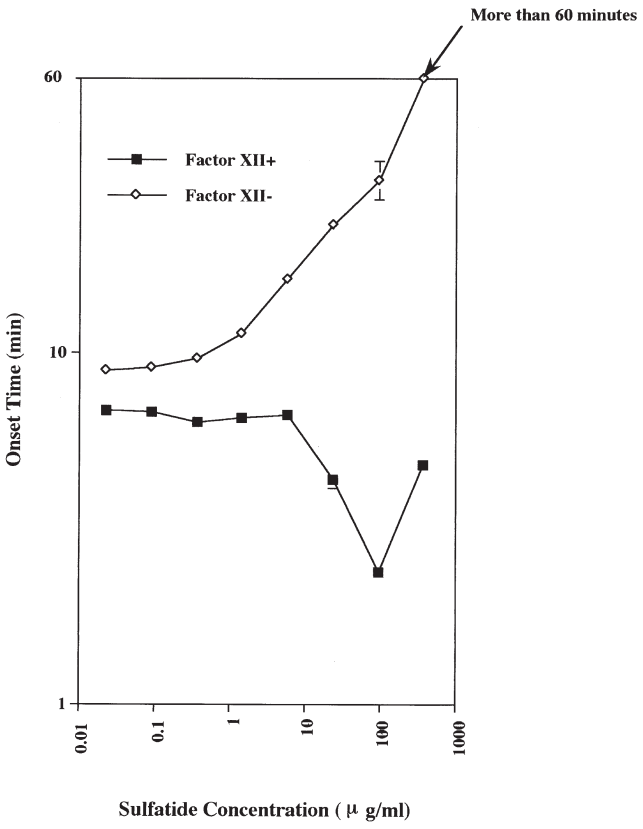

Figure 4. The relationship between sulfatide concentration [$\log_{10}$ (μg/ml)] and coagulation onset time [$\log_{10}$(min)] initiated by tissue factor in human normal and factor XII deficient plasma. In the XII-deficient plasma, at more than 0.37 μg/ml of sulfatide, the onset time was prolonged dose-dependently. In the normal plasma, a prolongation of coagulation time was not observed at 5.86 to 93.75 μg/ml of sulfatide, but the onset time was suddenly shortened with the increase of the concentration. At more than 93.75 μg/ml sulfatide, this shortening was abated. The symbols represent the means of triplicate experiments, and vertical bars represent SE.

Effect of sulfatide on PT test

Previously, we reported that sulfatide prolonged PT dose-dependently [10]. Since prolongation of PT usually reflects activities of anticoagulant agents [18], we reexamined the effect of sulfatide on the test very carefully. Figure 5 shows the relationship between the coagulation time and the concentration of sulfatide by PT test. In fact, sulfatide prolonged PT remarkably at more than 37.5 μg/ml, but at less than 18.8 μg/ml, on the contrary, it shortened PT slightly but definitely.

Discussion

We previously reported the natural occurrence of sulfatide in sera from various animals and that its content in human

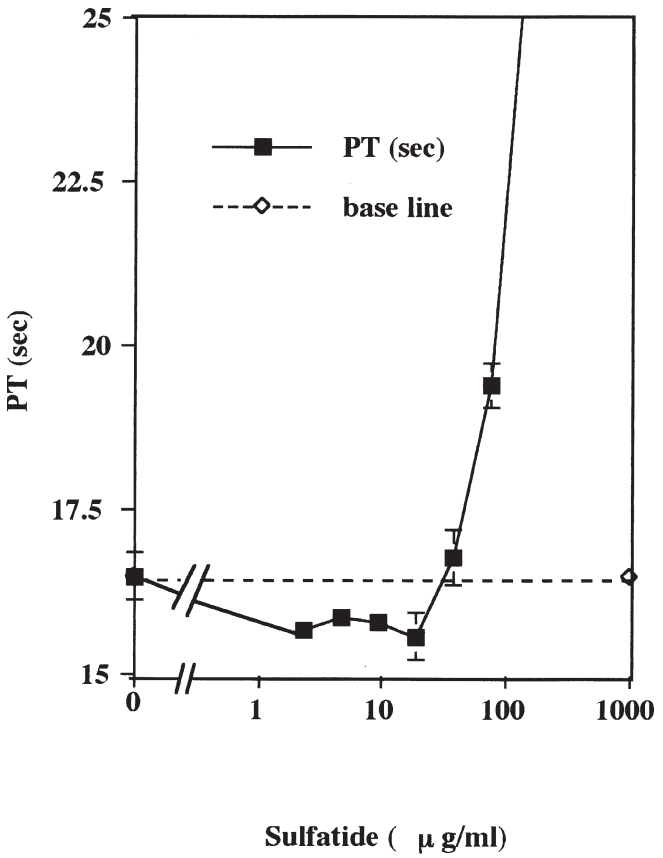

Figure 5. The relationship between coagulation time and sulfatide concentration by prothrombin time (PT) test, Sulfatide prolonged PT remarkably at more than 37.5 μg/ml, but at less than 18.8 μg/ml, it shortened PT slightly but definitely. The symbols represent the means of triplicate experiments, and vertical bars represent SE.

sera was 0.64 nmol/ml [9], which corresponds to 0.6 μg/ml when calculated based on the molecular weight (about 860) of sulfatide. Judging from the results of the present *in vitro* assay (Figure 3B), this content may be sufficient to enhance thrombogenesis in humans. The results of experiments with the DVT model imply that the thrombogenesis enhanced by sulfatide appears to require venous congestion and/or endothelial damage. Such situations may also possibly occur in humans under pathological conditions. For instance, cancer cells may invade vessels, resulting in an obstruction of the blood flow together with destruction of the endothelium. There have been many reports that cancer cells can induce thrombotic diseases. Although the major causes of such diseases have been attributed to procoagulant or coagulant activities, such as tissue factor or factor X activator produced by cancer cells, some uncharacterized factors have also been suggested [19]. From the standpoint of sulfatide, they should be reevaluated. Indeed, cancer cells producing and/or excreting sulfatide have been reported [20, 21].

Metachromatic leukodystrophy is a lysozomal disease, and the clinical symptoms are described as neurological abnormalities [22, 23]. A careful examination of the patients about coagulation system may be necessary since a large amount of sulfatide is accumulated in the patients because of the deficiency of arylsulfatase A.

Fibrin deposition in glomeruli is always observed in various types of glomerular diseases together with an accumulation of leukocytes which are believed to be responsible for producing coagulants such as tissue factor [24]. Fibrin deposition is also observed in the patients suffering from diabetic glomerulosclerosis despite unclear accumulation of leukocytes in the glomeruli [25]. Such fibrin formation may be related to sulfatide because its occurrence was suggested in diabetic glomeruli but not in normal glomeruli [26].

We previously reported that a large amount of sulfatide was accumulated in the atherosclerotic aortae of WHHL rabbits but not in the nonatherosclerotic aortae of normal rabbits, and we suggested its derivation from serum lipoprotein by analysis of the ceramide moiety in the sulfatide [12]. It has been suggested that continuous fibrin deposition accelerates the inflammatory process on the inner wall of the artery to contribute to atherosclerosis [27]. Thus, we should also reconsider atherosclerosis from the standpoint of sulfatide.

Although it may be possible that sulfatide works as a coagulant in some pathological conditions, the reverse possibility that sulfatide acts as an anticoagulant may still remain if the present *in vivo* observation could result of the activation of factor XII, as was found in the *in vitro* assay. Generally, hemostatic system via the extrinsic pathway (starting with tissue factor/factor VII) is believed to be physiologically important [17], but the role of the system via the intrinsic pathway, starting from contact activation, in which factor XII is involved, has been pointed out to be questionable because there is no bleeding symptom in patients lacking this factor [5–7]. As described in the result of *in vitro* assay, the factor XII-deficient plasma did not coagulate with or without sulfatide. In addition, when the factor XII-deficient plasma was stimulated by tissue factor, the plasma clotting time was prolonged dose-dependently in the presence of sulfatide probably due to a disturbance of fibrin formation by the binding of sulfatide to fibrinogen, as reported previously [10]. Indeed, we could not find thrombi in the rats when sulfatide was simply injected. Furthermore, some anticoagulant effect of sulfatide was suggested because a prolongation of bleeding time was observed after injection of sulfatide [10]. These observations suggested that sulfatide may require another cofactor to induce thrombosis *in vivo*, which could be induced in this rat experimental model by vein ligation and that otherwise sulfatide might still work as an anticoagulant if such a cofactor was inactivated. Sulfatide occurs naturally in plasma [9, 11] and the plasma membrane of many cells [28] including platelets [29]. In addition, sulfatide can bind many proteins such as laminin [29], thrombospondin [30], von Willebrand factor [31], antistatsin [32] and properdin [33]. Furthermore, it seems very important that most of such sulfatide-binding proteins have been found to be closely related to hemostasis, endothelial cells, and inflammation. These characteristics may be related to the enhancement of thrombogenesis. Because there is little knowledge on sulfatide participation in blood coagulation under various pathological conditions, we should examine hemostatic abnormalities more precisely under various pathological conditions, focusing on the elucidation of the role of this molecule.

Finally, recently anti-inflammatory actions of sulfatide have been reported and sulfatide may be expected as a promising anti-inflammatory medicine [34, 35]. However, sulfatide should be used carefully, because it may induce thrombosis under still unknown conditions.

Acknowledgments

We thank Dr. K. Takahashi (Shimane Medical College) and Dr. M. Waki (Seikagaku Corp. Tokyo Res. Inst.) for critical reading of the manuscript.

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Received 28 December 1997, revised 10 April 1998, accepted 13 April 1998