



Regular Article

Long duration of anticoagulant activity and protective effects of acharan sulfate in vivo

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ABSTRACT

Introduction: We previously reported that a new glycosaminoglycan, acharan sulfate (AS) from the African giant snail *Achatina fulica* showed anticoagulant activity in vitro, but was much less active when compared to heparin. In the present study, the anticoagulant activity of AS was investigated in vivo. **Methods:** AS and heparin were administered to mice and rats in various doses and the anticoagulant activities were measured by aPTT assay. Both were also compared in a thrombin-induced lethality animal model. As one of the possible mechanisms, AS-thrombin interaction was studied by using surface plasmon resonance spectroscopy. **Results:** Intravenous administration of AS to mice prolonged the clotting time (aPTT) in a time and dose-dependent manner. Although the anticoagulant activity was low in rats, it steadily increased over 5 h after administration of AS (30 mg/kg). In contrast, the increase in aPTT induced by 5 mg/kg of heparin was restored to a normal level after 3 h. In a thrombin-induced lethality model in mice, AS (20 mg/kg) protected against lethality by 80%, while heparin (20 mg/kg) did not show any protective activity beyond 3.5 h post-administration. AS could be also detected in plasma even 5 h after i.v. administration to rats. The binding constant (K_D) of AS to thrombin was 7.27×10^{-6} M, corresponding to moderate binding affinity. **Conclusions:** These results show that the longer duration of AS in blood could prolong the clotting time determined by aPTT and offering protection against thrombin-induced lethality. One possible mechanism may result from AS-thrombin interaction.

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Introduction

Acharan sulfate (AS) is a glycosaminoglycan (GAG) isolated from the giant African snail, *Achatina fulica*. This polysaccharide has a repeating disaccharide structure of $\rightarrow 4$ -2-deoxy-2-acetamide- α -D-glucopyranose (1 \rightarrow 4)-2-sulfo- α -L-idopyranosyluronic acid (1 \rightarrow [1,2] and its average molecular weight is about 135,000 Da, when determined by GPC-HPLC (gel-permeation chromatography-high performance liquid chromatography) analysis. Recently, we observed that AS interfered with heparin's bFGF mitogenicity in vitro [3] and it inhibits tumor growth in vivo [4]. Because AS structure is more closely related to heparin than to other glycosaminoglycans, we expected that AS might have in vivo anticoagulant activity like heparin. AS had been previously shown to exhibit weak anticoagulant activity in vitro [5]. However, in vitro results often correlate poorly with in vivo results, because they cannot mimic a myriad of hemodynamic and other unknown factors [6]. Therefore, in the present study, we examined the effect of thrombin-induced lethality as well as the anticoagulant activity of AS, when given intravenously to mice and rats. We also determined the plasma concentration of AS in rats after i.v. injection to assess its interactions with protein. As a first target, thrombin was chosen for the interaction with AS.

Materials and methods

Materials

AS was isolated from the giant African snail, *A. fulica* as previously described [1,2]. Heparin (porcine mucosa), chondroitin sulfate (MW 12000, bovine trachea), and heparin lyase II were purchased from Sigma (St. Louis, MO, USA). Human thrombin was kindly provided by Green Cross Pharmaceutical (Seoul, Korea). The reagents for the measurement of activated partial thromboplastin time (aPTT) were also obtained from Sigma. Alcalase was obtained from Novo Korea (Seoul, Korea). Centrifugal filters (Biomax-5) having a molecular weight cut-off of 5000 were purchased from Millipore (Bedford, MA, USA). Sensor chip CM-5, *N*-hydroxysuccinimide (NHS), and *N*-ethyl-*N*-(dimethylaminopropyl)-carbodiimide (EDC) were from BIAcore AB (Uppsala, Sweden).

Animals

Male Sprague–Dawley rats (300–350 g) and male ICR mice (26–32 g) were used in the experiments. The animals supplied from the Animal Breeding Center of Seoul National University were housed at 22 ± 1 °C, 10% humidity in a 12 h light–dark cycle. A commercial solid food (Samyang Yuji, Seoul, Korea) and tap water were given ad libitum. All animal work was carried out in accordance with the procedure outlined in the Guide for the Care and Use of Laboratory Animals. The volume of i.v. administration was 5 or 10 ml/kg of body weight in rats or mice, respectively.

Extraction and determination of acharan sulfate in plasma

AS in plasma was isolated according to the previous report with slight modifications [7]. Briefly, a portion (25 μ l) of rat plasma was combined with 20 μ l of 0.05 M Tris–HCl buffer (pH 8.0) containing 1 % (w/w) actinase E (Kaken Pharmaceutical, Tokyo, Japan) and incubated at 45 °C for 3 h to digest plasma proteins. Acetic acid (200 μ l of 15 mM) containing 10% NaCl was added to the reaction mixture and boiled for 5 min. The mixture was cooled on ice and centrifuged at $2300 \times g$ for 15 min, and the supernatant was transferred to another test tube, and a 180 μ l of supernatant was transferred to a centrifugal filter containing 20 μ l of 0.1 M NaOH and centrifuged as above. After centrifugation the retained AS fraction was washed twice with 0.2 M Tris–HCl buffer (pH 8.0). A 300 μ l of 0.1 M acetate buffer (pH 7.0) containing 10 mM calcium acetate was added and centrifuged ($2300 \times g$ for 15 min). The sample was digested with 10 μ l; of 5 mU heparin lyase II at 37 °C for 3 h. Finally, the sample was analyzed by the post-column derivatization HPLC as previously described [8]. In an alternative experiment, 100 μ l of human plasma was added to tubes containing 100 μ l of AS solution (20 mg/ml in 50 mM Tris–HCl buffer, pH 8.0) and incubated for 0, 0.5, 1, 2, 3 and 5 h at 37 °C. After each incubation time, 200 μ l of 50 mM Tris–HCl buffer (pH 8.0) containing 1% actinase E was added and incubated at 45 °C for 3 h. The solution was extracted with 400 μ l of phenol/chloroform/isoamyl alcohol (25:24:1, Sigma) and the aqueous phase was analyzed by GPC-HPLC as previously described [5].

Administration of samples to laboratory animals

The test samples were dissolved in saline and given i.v. into tail veins under a mild anesthesia

by ether: (i) four groups of mice received AS solution at the doses of 0 (saline), 1.1, 3.3, and 10 mg/kg, one hour later, blood was collected; (ii) three groups of mice received AS or heparin at a dose of 10 mg/kg, or saline, and then blood was collected at 0.5, 1, 2, and 3 h after administration of the samples; (iii) three groups of rats received AS or heparin at doses of 30 and 5 mg/kg or saline, respectively. At regular intervals (at 0.5, 1, 2, 3, and 5 h) after administration, a 0.2 or 0.5 ml citrated (1:10 dilution, 3.8% sodium citrate) blood sample was collected by puncturing the retro-orbital venous plexus under mild ethyl ether anesthesia in mice or rats, respectively. Platelet-poor plasma was immediately prepared by centrifugation ($1870 \times g$, 10 min, MICRO 17R, Korea), and then aPTT was measured according to the following procedure. The activity was compared with the saline and heparin groups.

Measurement of aPTT

The aPTT was measured with a fibrometer (BBL Fibrosystem, Becton Dickinson, Cockeysville, MD, USA). For the aPTT measurement of in vitro samples, 100 μ l of human plasma was mixed with 100 μ l of sample dilutions followed by the addition of 100 μ l aPTT reagent (Sigma), and 100 μ l of 0.035 M calcium chloride to start clot formation. Ex vivo samples were tested in a similar way.

Protective effect in thrombin-induced lethality in mice

Thrombin (20 units/mouse) was i.v. given to mice at 10 min, 3.5 h or 5 h after the i.v. administration of test samples (heparin, AS, and chondroitin sulfate) [9]. Thereafter, the behavior and lethality of treated mice were observed.

Immobilization of thrombin to sensor chip

Thrombin was covalently immobilized to the biosensor surface through its primary amine groups. First, carboxymethyl groups at the CM 5 chip surface were activated using an injection pulse of 50 μ l (flow rate, 5 μ l/min) of an equimolar mixture of NHS/EDC (final concentration 0.05 M, mixed immediately before injection). Then, 40 μ l of thrombin (100 μ g/ml in 10 mM sodium acetate buffer) was applied. Excess unreacted sites on the sensor surface were blocked with a 50- μ l injection of 1 M ethanolamine. To prepare the control flow cell, bovine serum albumin was

immobilized on the surface using a similar coupling procedure.

Kinetic measurement of AS interaction with immobilized thrombin by surface plasmon resonance spectroscopy (SPR)

AS in HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% polysorbate 20 (v/v), pH 7.4) was injected over thrombin and control surfaces at different concentrations at a flow rate of 5 μ l/min. At the end of sample injection, the same buffer was flown over the sensor surface to facilitate dissociation studies. After a suitable dissociation time, the sensor surface was regenerated by injecting with 1 M NaCl/50 mM NaOH. Responses were monitored as a function of time (sensorgram) at 25 °C on BIAcore 2000 (BIAcore AB, Uppsala, Sweden). Apparent kinetic parameters were evaluated using the BIAcore 2000 software.

Statistical analysis

Data were expressed as means \pm S.D. To analyze the data statistically, we performed one-way analysis of variance (ANOVA) for repeated measurements of the same variable, and used Duncan's multiple range *t*-test to determine which means were significantly different from that of the control. We considered differences significant at * $p < 0.05$ and ** $p < 0.01$.

Results

Dose-dependency of acharan sulfate on aPTT in mice

The aPTT of mice at different doses of AS is shown in Fig. 1. Intravenous administration of AS prolonged aPTT in a dose-dependent manner 1 h after the sample treatment. Clotting times on administration of 3.3 and 10 mg/kg were markedly increased compared to the control. The data suggest that AS can interact with some of the proteins associated with anticoagulation.

Time-dependency of acharan sulfate on aPTT in mice and rats

The effect on aPTT at different time intervals after administration of a dose of 10 mg/kg AS is shown in Fig. 2. After i.v. administration of AS to

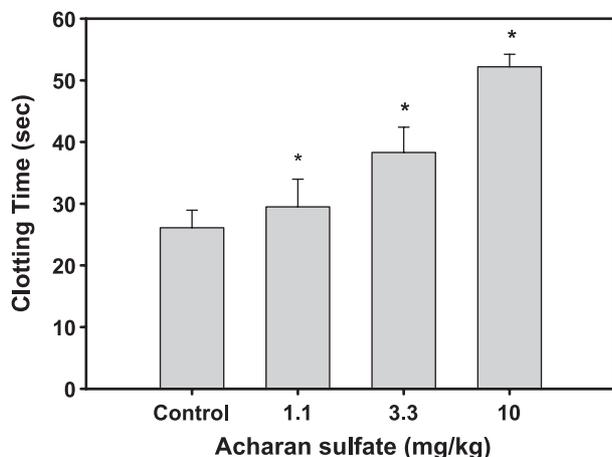


Fig. 1 Dose-dependency of acharan sulfate on activated partial thromboplastin time in mice. Acharan sulfate was intravenously administered to mice at doses of 1.1, 3.3, and 10 mg/kg. Blood samples were taken out 90 min after administration, and then aPTT was measured ($n=8$, mean \pm SD). * $p<0.05$.

mice, the plasma aPTT increased up to ~ 2.3 -fold during the first 30 min and then decreased to ~ 1.6 -fold of the control value and maintained this level over a 3 h period. Similarly, the aPTT was obtained in rats receiving a dose of 30 mg/kg AS and the data is shown in Fig. 3. After i.v. administration of 30 mg/kg AS to rats, the aPTT increased up to ~ 1.5 -fold and was maintained over a period of 5 h. In contrast, the aPTT at dose of 5 mg/kg heparin was mark-

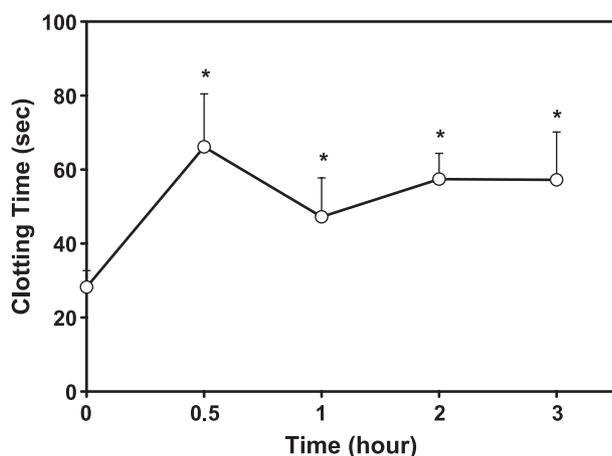


Fig. 2 Time-dependency of acharan sulfate on activated partial thromboplastin time in mice. Acharan sulfate was intravenously administered to mice at a dose of 10 mg/kg. At different time intervals after administration of AS, blood samples were taken out and aPTT was assayed ($n=8$, mean \pm SD). * $p<0.05$.

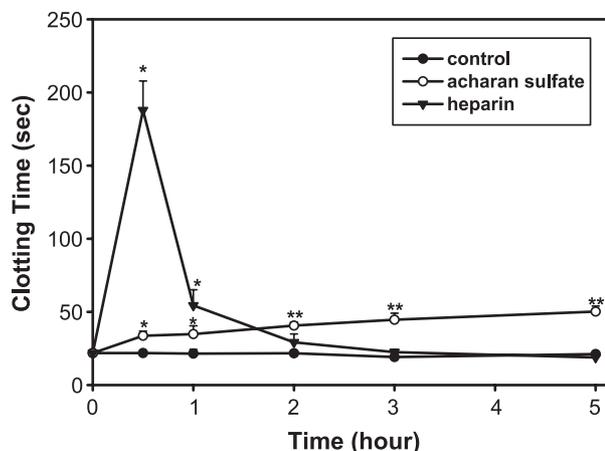


Fig. 3 Ex vivo anticoagulant effects of acharan sulfate and heparin in rats. Acharan sulfate (30 mg/kg) and heparin (5 mg/kg) were intravenously administered to rats. At different time intervals after administration of AS, blood samples were taken out and plasma was prepared, and then aPTT was determined ($n=5$, mean \pm SD). * $p<0.05$ and ** $p<0.01$.

edly elevated to 200 s for 30 min, but rapidly decreased to normal values after 2 h.

Protective effect on thrombin-induced lethality in mice

As shown in Table 1, injection of thrombin at dose of 20 units/mouse caused 90–100% lethality. Pretreatment with AS (20 mg/kg) only protected 50% of the animals 10 min after thrombin-induced lethality, but 70–80% of mice were alive when AS was

Table 1 Effect of glycosaminoglycans on thrombin-induced lethality in mice

Sample	Dose (mg/kg)	Injection time of thrombin after treatment of drugs	No. of mice	Survival	Survival rate (%)
Saline		10 min	10	0	0
Heparin ^a	5	10 min	10	10	100
	5	3 h 30 min	10	0	0
	20	5 h	10	0	0
AS	20	10 min	10	5	50
		3h 30 min	10	8	80
		5 h	10	8	80
CS	20	5 h	10	1	10

^a The treatment was done separately. One group received heparin (5 mg/kg) and lethality was measured at 10 min and 3 h 30 min, respectively. The other group received heparin (20 mg/kg) and lethality was measured at 5 h.

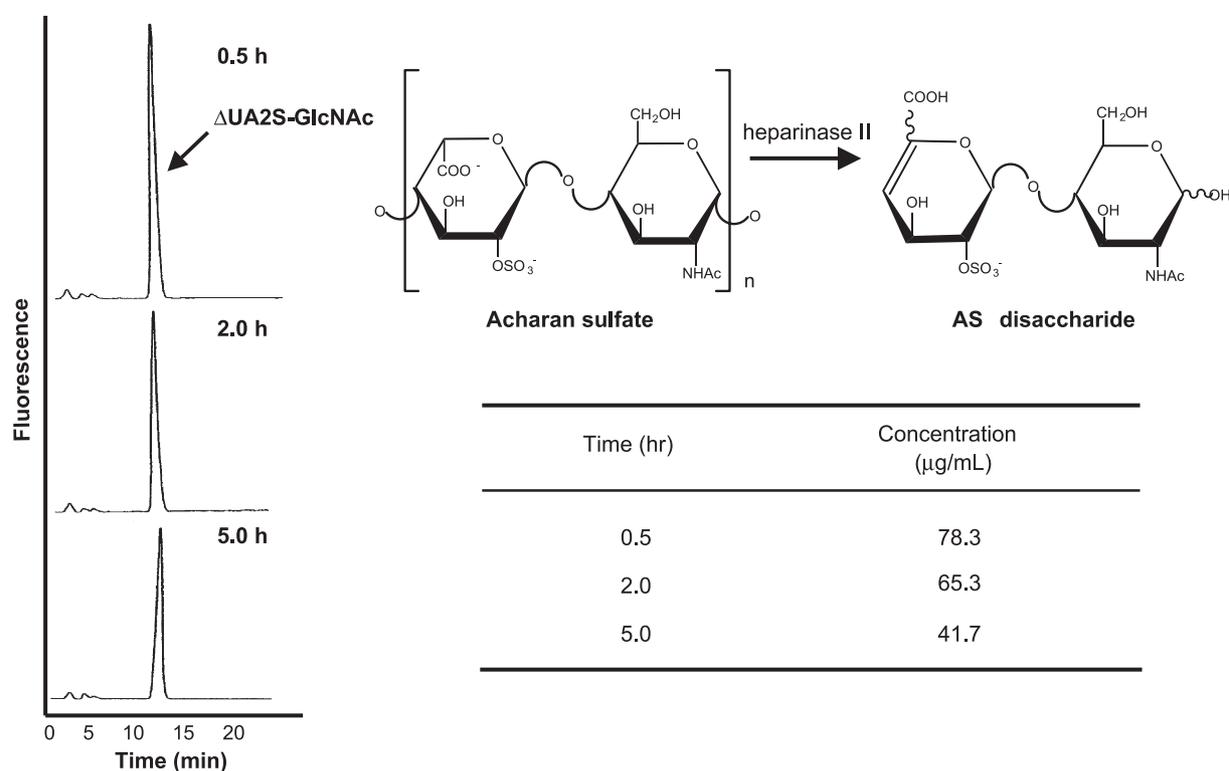


Fig. 4 HPLC analysis of AS disaccharide (Δ UA2S-GlcNAc) after enzymatic depolymerization of acharan sulfate obtained from rat plasma.

given at 3.5, and even 5 h prior to challenge thrombin. Heparin at a dose of 5 mg/kg protected 100% when given 10 min before a lethal dose of thrombin. However, heparin lost its ability to protect when given 3.5 h before injection of thrombin. Heparin (or chondroitin sulfate) at a dose of 20 mg/kg also showed no protective activity when given 5 h prior to challenge with thrombin (Table 1). These results are consistent with the prolonged lifetime of AS, compared to heparin, as measured by aPTT in rats (Fig. 3).

Determination of acharan sulfate concentration in rat plasma

The plasma concentration of AS was determined based on the unsaturated AS-derived disaccharide after enzymatic cleavage as shown in Fig. 4. The concentration reached the highest peak at 0.5 h after intravenous injection and then it decreased slowly. To examine whether AS was being degraded in plasma, intact AS was incubated in human plasma for 5 h and then extracted with chloroform-phenol-isoamylalcohol. The recovered AS was analyzed by GPC-HPLC. No difference of molecular weight was observed following incubation of AS in plasma (data

not shown). These results suggest that AS was not degraded in plasma even on 5 h incubation.

Immobilization of thrombin on biosensor chip CM-5

A sensor chip CM-5 coated with a carboxymethyl dextran was used for interaction analysis. Amine

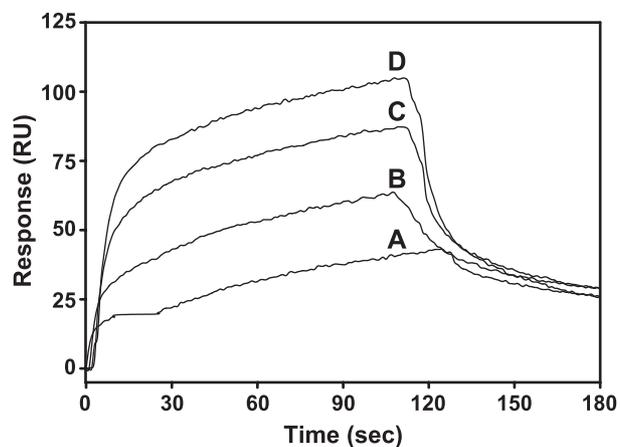


Fig. 5 Sensorgrams of the interaction between thrombin and AS. (A) 1 μ M (B) 2 μ M (C) 4 μ M (D) 6 μ M AS.

coupling is the most common choice for immobilization of proteins onto biosensor chips. After coupling, ethanolamine was added to deactivate remaining active ester [10]. Successful immobilization of thrombin was confirmed by the observation of a ~ 5000 RU (resonance signal) response.

Interaction of acharan sulfate with thrombin

Sensorgrams were fitted using a Langmuir 1:1 binding model, which showed thrombin and AS interacted (Fig. 5). The nonspecific binding to the flow cell was removed by subtracting the binding data from a control cell containing immobilized albumin. The kinetic parameters calculated from the binding data are: k_{on} (on rate constant) = 1.25×10^3 ($M^{-1} s^{-1}$), k_{off} (off rate constant) = 9.12×10^{-3} (s^{-1}), and $K_D = 7.27 \times 10^{-6}$ M. The μM value of the calculated K_D for the thrombin-AS complex demonstrates a moderately strong binding affinity between these molecules.

Addition of 10 and 30 μg AS to human plasma prolonged the aPTT by 2–2.5-fold (Fig. 6). Exogenous thrombin (3 mU) in plasma promoted clotting time by 2-fold. When both 3 mU thrombin and AS (10 and 30 μg) were incubated together, AS blocked the coagulant activity of thrombin (Fig. 6). The results of binding experiment and aPTT measurement strongly suggest that AS can interact with thrombin affecting the thrombin-induced coagulation.

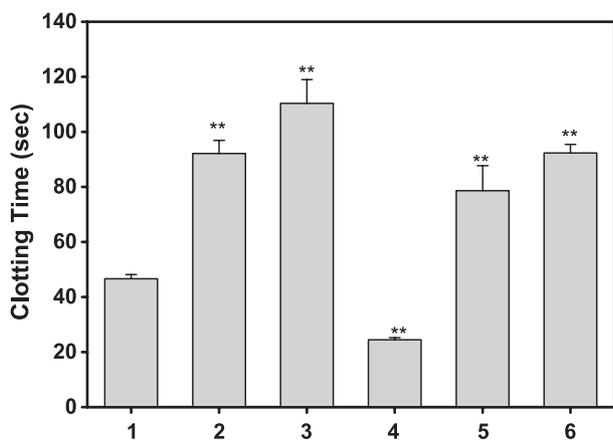


Fig. 6 aPTT measurement in the presence of exogenous AS and thrombin. The aPTT was measured by adding thrombin (3 mU) and AS (10 and 30 μg) to human plasma ($n=3$, mean \pm SD). (1) Control plasma; (2) plasma + 10 μg AS; (3) plasma + 30 μg AS; (4) plasma + 3 mU thrombin; (5) plasma + 3 mU thrombin + 10 μg AS; (6) plasma + 3 mU thrombin + 30 μg AS.

Discussion

Acharan sulfate (AS) is a glycosaminoglycan and the structure is similar to heparin and heparan sulfate. In this report, we demonstrated that the anticoagulant efficacy of AS *in vivo* was maintained a longer time than by heparin.

It is reported that the half-life of heparin is dependent on the dose [11]. In the present study, we investigated the activity of heparin at doses of 5 mg/kg in rats, 5 and 20 mg/kg in mice, respectively. Our results showed that AS was quite different from heparin in that it did not have a narrow concentration peak. Instead, it maintained a long plateau phase based on aPTT assay in mice and rats. Unlike heparin, the clotting time increased more at 5 h than at 0.5 h post-administration in rats. The measurement of aPTT indicated that AS remained in rat blood over 5 h after treatment and its effect decreased only gradually. The observed dependence between concentration and anticoagulant suggests that it binds to some proteins important for anticoagulation. To confirm the long-lasting efficacy of AS, we also investigated the antithrombotic activity using a thrombin-induced lethality model in mice. Previous reports indicated that chondroitin sulfate remained in the blood for an extended time after its administration, when it was given orally or intramuscularly [12–14]. Therefore, we compared the protection of lethality by heparin and chondroitin sulfate on this model. The results showed that the pretreatment of AS (20 mg/kg) moderately protected against lethality by 50% when it was given at 10 min before thrombin injection. Surprisingly, AS could protect against lethality by 70–80% when it was given at 3.5 and 5 h prior to a thrombin challenge. In contrast, neither heparin nor chondroitin sulfate showed this protective activity at 3.5 or 5 h after administration. Heparin, however, could markedly protect against lethality when administered 10 min prior to challenge by thrombin. Thrombin mediates a variety of critical biological processes, such as signaling in platelets in hemostasis and thrombosis [15]. Since 2-sulfo-iduronic acid residues of AS reside in the 1C_4 or 2S_0 conformations [16], this flexible structure may enhance its binding to proteins. Such interactions with plasma proteins may be ascribed to a variety of biological functions under physiological conditions. Because AS anticoagulant activity was higher in *ex vivo* studies, binding experiment was performed using a SPR biosensor, which has become an established method of measuring molecular interactions [17]. Binding of AS to thrombin was specific and caused a

lowering clotting time induced by thrombin. AS may bind to some plasma proteins, such as thrombin, in a different way than does heparin. The aPTT in the presence of exogenous thrombin was increased by addition of AS to plasma. This strongly suggests that thrombin interacts with AS in either a specific or nonspecific fashion. The surface of thrombin contains patches of positively charged amino acids, i.e. anion exosite I and II. Exosite II contains a large number of basic amino acids, such as arginines and lysines, and has been identified as the heparin-binding site [11,15,18]. AS might also bind to this exosite in a similar way as heparin. Because AS contains only 2-sulfo-iduronic and *N*-acetylglucosamine residues, it lacks the glucuronic acid and 3,6-*O*-sulfo groups in glucosamine residues critically important for binding antithrombin III [19]. Therefore, the longer duration of AS in plasma could be attributed to its binding plasma proteins, such as thrombin, in a different way from heparin-binding to these same proteins.

Acknowledgements

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