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Dermatan sulfate is the predominant antithrombotic glycosaminoglycan in vessel walls: Implications for a possible physiological function of heparin cofactor II

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Abstract

The role of different glycosaminoglycan species from the vessel walls as physiological antithrombotic agents remains controversial. To further investigate this aspect we extracted glycosaminoglycans from human thoracic aorta and saphenous vein. The different species were highly purified and their anticoagulant and antithrombotic activities tested by in vitro and in vivo assays. We observed that dermatan sulfate is the major anticoagulant and antithrombotic among the vessel wall glycosaminoglycans while the bulk of heparan sulfate is a poorly sulfated glycosaminoglycan, devoid of anticoagulant and antithrombotic activities. Minor amounts of particular a heparan sulfate (<5% of the total arterial glycosaminoglycans) with high anticoagulant activity were also observed, as assessed by its retention on an antithrombin-affinity column. Possibly, this anticoagulant heparan sulfate originates from the endothelial cells and may exert a significant physiological role due to its location in the interface between the vessel wall and the blood. In view of these results we discuss a possible balance between the two glycosaminoglycan-dependent anticoagulant pathways present in the vascular wall. One is based on antithrombin activation by the heparan sulfate expressed by the endothelial cells. The other, which may assume special relevance after vascular endothelial injury, is based on heparin cofactor II activation by the dermatan sulfate proteoglycans synthesized by cells from the subendothelial layer.

Keywords: Vascular glycosaminoglycan; Anticoagulant glycosaminoglycan; Thrombosis; Dermatan sulfate; Heparan sulfate; Heparin cofactor II

1. Introduction

Antithrombin is the major plasma anticoagulant cofactor that neutralizes the intrinsic proteases of the coagulation system. Mutations that reduce the level of antithrombin activity predispose patients to venous thrombosis [1]. Complete antithrombin deficiency is incompatible with human life and in mice causes intrauterine death from an extreme hypercoagulable state [2,3]. The rate of protease neutralization by antithrombin is dramatically enhanced by heparin [4], a polysaccharide similar to heparan sulfate. It has been hypothesized that heparan sulfate on the endothelial cell surface might similarly accelerate antithrombin activity and thereby contribute to the non-thrombogenic properties of blood vessels [5]. However, it is presently unclear whether heparan sulfate from blood vessels is a major physiological modulator of hemostasis. The major clue to this point came from the observation that mice deficient in 3-*O*-sulfotransferase, an enzyme responsible for sulfation of a heparan sulfate region with high affinity for antithrombin, have a normal phenotype and have the same amount of fibrin deposition in their tissues as wild-type mice [6]. These findings raise the possibility that another glycosaminoglycan can compensate for the reduction in heparan sulfate activity.

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Heparin cofactor II is another anticoagulant cofactor found in plasma [7]. It only inactivates thrombin whereas antithrombin inactivates other coagulation enzymes including factor Xa and IXa [8]. Dermatan sulfate, a glycosaminoglycan found in the extracellular matrix (including the arterial and venous walls), activates heparin cofactor II, but has no effect on antithrombin [9]. Several patients with inherited heparin cofactor II deficiency and histories of venous thromboembolic disease have been reported [10,11]. In contrast to this observation, heparin cofactor II-deficient mice undergo normal life with no evidence of thrombosis or bleeding secondary to disseminated intravascular coagulation. However, in comparison to wild-type animals, these mice show a shorter thrombotic occlusion time of the carotid artery after endothelial cell injury [12], indicating that heparin cofactor II contributes to the non-thrombogenic properties of blood vessels. Although dermatan sulfate occurs in high amounts in vessel walls [13,14], it has not been investigated in detail whether this glycosaminoglycan from arterial or venous walls can prevent thrombosis on in vivo experiments.

Here we used an alternative approach to investigate the possible contribution of the various glycosaminoglycan species to the non-thrombogenic properties of blood vessels. The glycosaminoglycans were extracted from the vessel walls, highly purified, and their anticoagulant and antithrombotic activities tested on in vitro and in vivo assays, respectively. Dermatan sulfate is the major anticoagulant and antithrombotic glycosaminoglycan in the vessel walls. We suggest that heparin cofactor II activation by vascular wall dermatan sulfate is an anticoagulant pathway, which assumes special relevance under pathological conditions, after endothelial injury.

2. Materials and methods

2.1. Isolation and purification of vessel wall glycosaminoglycans

Human thoracic aortas were obtained at necropsy and normal saphenous veins isolated from patients undergoing coronary bypass surgery. All the samples were conserved in acetone until used. In the aorta samples, after removing the adventitial layer, only the segments containing intima+media layers devoid of macroscopically visible atherosclerotic lesions were used for glycosaminoglycan extraction. Samples were cut into small pieces and subject to two changes of 10 volumes of chloroform:methanol (2:1, vol/vol) for 24 h each. The final defatted powder was obtained by drying this material under vacuum.

The chloroform/methanol-treated venous or arterial powder (100 mg, dry weight) was re-hydrated in 3.7 mL of 0.1 M sodium acetate buffer (pH 5.0), containing 5 mM cysteine and 5 mM EDTA at 4 °C for 24 h. Thereafter, total glycosaminoglycans were isolated by proteolysis with papain, followed by subsequent cetylpiridinium and ethanol precipitations, as described previously [13,15]. The final pellet was dried under vacuum and dissolved in 1.0 mL distilled water. Total glycosaminoglycan contents in the samples were estimated by determination of hexuronic acid concentration of the final solutions [16]. Under these conditions, papain digestion completely dissolved all vessel samples, and controls using known amounts of glycosaminoglycans showed that recovery from the subsequent cetylpyridinium and ethanol precipitation was greater than 90%.

Fractionation of the glycosaminoglycans was performed by anion exchange chromatography on a Mono Q-FPLC column. The eluted fractions were assayed by the metachromasy produced by sulfated glycosaminoglycans with 1,9-dimethylmethylene blue [17] and by hexuronic acid content using the carbazole reaction [16]. Fractions were pooled, dialyzed against distilled water, lyophilized and then dissolved in distilled water. The purity of each fraction was checked by agarose gel electrophoresis, digestion with specific glycosaminoglycan lyases and deamination with nitrous acid, as described [13–15].

In some experiments the heparan sulfate and chondroitin sulfate+dermatan sulfate fractions were obtained by digestion with chondroitin ABC lyase and deamination with nitrous acid, respectively.

2.2. Disaccharide analysis following enzymatic depolymerization of the aortic glycosaminoglycans

Purified chondroitin sulfate+dermatan sulfate fraction (~100 μg) was either digested with 0.01 unit of chondroitin AC lyase from *Arthrobacter aurescens* or chondroitin ABC lyase from *Proteus vulgaris* (both from Seikagaku America Inc.) in 100 μL of 0.05 M Tris/HCl (pH 8.0), containing 5 mM EDTA and 15 mM sodium acetate. After incubation at 37 °C for 12 h, the unsaturated disaccharides formed by the enzymatic digestions were analyzed on a SAX-HPLC analytic column, as described [18]. The unsaturated disaccharides formed by chondroitin AC lyase digestion allow us to determine the proportions of disaccharide units in the chondroitin sulfate chain. As the unsaturated disaccharide formed from dermatan sulfate and chondroitin 4-sulfate by the action of chondroitin AC and ABC lyases is the same, namely α-ΔUA-GalNAc($4SO_4$), and dermatan sulfate is resistant to the action of the chondroitin AC lyase, the difference between the amount of α-ΔUA-GalNAc($4SO_4$) formed by chondroitin AC and by ABC lyases enables us to estimate the amount of disaccharide units containing iduronic acid in the mixture.

Heparan sulfate fraction (~200 μg) was exhaustively digested with a mixture of heparin and heparan sulfate lyases. The released disaccharides were analyzed as described [18] and identified by comparison with the

elution of known disaccharide standards obtained from Seikagaku America Inc.

2.3. Anticoagulant action measured by APTT

Activated partial thromboplastin time (APTT) clotting assay was performed according to the manufacturer's specifications, as described [19]. In this assay, normal human plasma (90 μL) were mixed with different amounts of glycosaminoglycan (0→50 μg as hexuronic acid per assay) in 0.9% NaCl (10 μL), warmed for 60 s at 37 °C and then 100 μL of prewarmed APTT reagent (Biolab-Merieux AS, Rio de Janeiro, Brazil) was added and allowed to incubate for 2 min at 37 °C. Pre-warmed 0.25 M calcium chloride (100 μL) was then added and the APTT was recorded as the time for clot formation in a coagulometer (Amelung KC4A). The anticoagulant activity of the chondroitin sulfate+dermatan sulfate and heparan sulfate fractions were expressed as international units (IU)/mg based on a parallel standard curve with the 4th International Standard Heparin (190 IU/mg).

2.4. Inhibition of thrombin by antithrombin or heparin cofactor II in the presence of glycosaminoglycan

Incubations were performed in disposable semi-micro-cuvettes. The final concentrations of reactants included 100 nM antithrombin or 150 nM heparin cofactor II, 20 nM thrombin and 0→100 μg as hexuronic acid/mL of the glycosaminoglycan from the artery or vein in 100 μL TS/PEG buffer (0.02 M Tris–HCl, 0.15 M NaCl and 1.0 mg/mL polyethylene glycol, pH 7.4). Thrombin was added last to initiate the reaction. After 60-s-incubation at room temperature, 500 μL of TS/PEG buffer containing 100 μM chromogenic substrate S-2238 for thrombin (Chromogenix AB, Mondal, Sweden) was added, and the absorbance at 405 nm was recorded for 120 s. The rate of change of absorbance was proportional to the thrombin activity remaining in the incubation. No inhibition occurred under our experimental conditions when thrombin was incubated with antithrombin or heparin cofactor II in the absence of glycosaminoglycan. In addition, no inhibition was observed when thrombin was incubated with glycosaminoglycan alone over the range of concentrations tested.

2.5. Assessment of antithrombotic properties

Antithrombotic activity was investigated in rats with brain thromboplastin as the thrombogenic stimulus [20]. We followed the institutional guidelines for animal care and experimentation. Briefly, rats (both sexes, ~200 g body weight) were anaesthetized with an intramuscular injection of 100 mg/Kg body weight of ketamine (a kind gift by Cristália, São Paulo, Brazil) and 16 mg/Kg body weight of xylazine (Bayer AS, São Paulo, SP). Inferior cava vein was isolated and the right carotid artery cannulated. Different doses of the arterial glycosaminoglycan were injected into the right carotid artery and allowed to circulate for 3 min. Then, brain thromboplastin (5 mg/kg body weight) from Biolab-Merieux AS (Rio de Janeiro, Brazil) was slowly injected intravenously and after 1 min 0.7 cm of isolated cava vein was clamped off using distal and proximal sutures. A high dose of thromboplastin was used in order to decrease the platelet activation role in the development of venous thrombosis [21]. After 20 min stasis, the thrombus formed inside the occluded segment was carefully pulled out, washed with 5% sodium citrate, dried for 1 h at 60 °C and weighted. Thrombus weight as mg (mean ± SEM) was expressed at various glycosaminoglycan doses (mg or IU/Kg body weight).

2.6. Antithrombin-affinity chromatography

An antithrombin-affinity column was prepared as described by Höök et al. [22]. Briefly, 20 mg of antithrombin in 5 mL of coupling buffer (50 mM NaCl, 100 mM $NaHCO_3$, pH 8.2) were mixed with 240 mg of *N*-acetylated heparin (120 mg/mL in 100 mM $NaHCO_3$, pH 8.2) to block free amino groups in the glycosaminoglycan-binding sites of the antithrombin molecules. HiTrap NHS-activated HP (2 mL) from Amersham Biosciences was combined with the antithrombin/heparin solution and coupling was carried out as described by the manufacturer. The immobilized antithrombin maintains full heparin-binding activity, since it produces two distinct heparin fractions that differ markedly in their anticoagulant activities, estimated as 432 and ~20 IU/mg, respectively, based on the APTT assay. Glycosaminoglycans extracted from human thoracic aorta (4 mg) and standard heparin from porcine intestinal mucosa (200 μg) were applied to 1 mL of antithrombin-affinity column, pre-equilibrated with 20 mM Tris/HCl (pH 7.4) and connected to an FPLC system from Amersham Biosciences. The column was washed with 10 mL of the same Tris solution and eluted at a flow rate of 0.5 mL/min using a linear NaCl gradient of 0.15→3.0 M NaCl. Fractions of 0.5 mL were collected and analyzed for metachromatic property using 1,9-dimethylmethylene blue [17]. The NaCl concentration was estimated by conductivity and the range of salt concentrations used in our experiments did not interfere with the metachromasy assay. The fractions of glycosaminoglycans obtained on the antithrombin-affinity column were identified by agarose gel electrophoresis, as described [13,14].

2.7. Statistical analysis

Results are expressed as mean standard errors of the mean. The difference between two groups was tested using the Mann-Whitney *U* test.

3. Results

3.1. Vessel heparan sulfate is a poorly sulfated glycosaminoglycan

Aortic glycosaminoglycans were analyzed by anion-exchange chromatography on a Mono Q-FPLC. On elution with a linear gradient of NaCl, the aortic heparan sulfate showed a single peak eluted at ~0.7 M NaCl (open circles in Fig. 1A), clearly at a lower salt concentration than that required for the elution of standard heparin (~1.2 M, closed circles in Fig. 1A) or of aortic chondroitin sulfate+dermatan sulfate (~1.0 M in Fig. 1B). It is not possible to separate the mixture of aortic chondroitin sulfate and dermatan sulfate using anion exchange chromatography, only a partial separation is achieved on gel filtration column. The degree of sulfation and the distribution of the sulfate esters in the arterial glycosaminoglycans were ascertained by the pattern of degradation products formed after digestion with specific

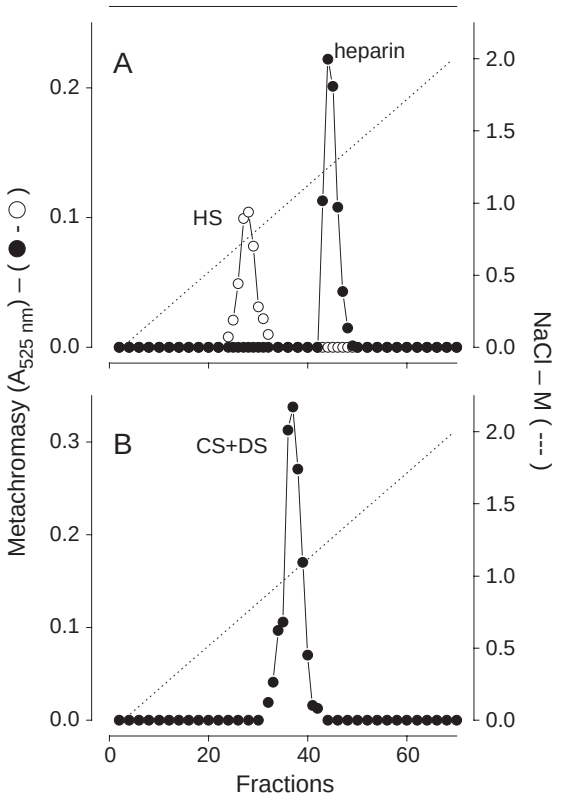

Fig. 1. Anion-exchange chromatography of the aortic glycosaminoglycans on Mono Q-FPLC. Purified heparan sulfate (HS, O in Panel A) and chondroitin sulfate+dermatan sulfate (CS+DS, ● in Panel B) from human thoracic aorta and a heparin standard (● in Panel A) (2.0 mg of each) were applied to a Mono Q-FPLC column (HR 5/5) equilibrated with 20 mM Tris–HCl buffer (pH 8.0). The column was developed by a linear gradient of 0→2.0 M NaCl in the same buffer. The flow rate of the column was 0.5 mL/min, fractions of 0.5 ml were collected and assayed by the metachromasy produced with 1,9-dimethylmethylene blue. The concentrations of NaCl were estimated by conductivity of the solutions. Arterial and venous glycosaminoglycans do not differ in their elution profile on a Mono Q column [14].

Table 1
Disaccharide composition and sulfation characteristic of heparan sulfate and chondroitin sulfate+dermatan sulfate from thoracic human aorta

Glycosaminoglycan	Disaccharide unit[a]	%	Sulfate/100 disaccharides
Heparan sulfate[b]	α-ΔUA-GlcNAc	45	
	α-ΔUA-GlcNAc(6SO_4)	16	
	α-ΔUA-GlcNSO_4	25	
	α-ΔUA-GlcNSO_4(6SO_4)	15	
	NSO_4		40
	OSO_4		31
	Total SO_4		71
Chondroitin sulfate + Dermatan sulfate[c]	GlcUA-GalNAc4SO_4	13	
	GlcUA-GalNAc6SO_4	50	
	IdUA-GalNAc4SO_4	37	
	OSO_4		100

[a] α-ΔUA indicates α-$\Delta^{4,5}$-unsaturated hexuronic acid, GlcNS; *N*-sulfated glucosamine; 6S, 6-sulfated; GlcNAc, *N*-acetyl-glucosamine; GlcUA, glucuronic acid; GalNAc, *N*-acetyl-galactosamine and IdUA, iduronic acid.
[b] Relative proportions of disaccharide units were determined by digestion of the purified heparan sulfate fraction with a mixture of heparin and heparan sulfate lyases. Minor components (<5%) are not reported.
[c] Relative proportion of disaccharide units were determined by degradation with chondroitin AC and ABC lyases of the dermatan sulfate+chondroitin sulfate fraction. Minor components (<5%) are not reported. See also Methods.

lyases (Table 1). Again, aortic heparan sulfate has a lower sulfation degree (~71 sulfate groups/100 disaccharides) when compared to the same glycosaminoglycan from other sources. Highly sulfated heparan sulfate with ~133 sulfate groups/100 disaccharides have been isolated from several other sources [18,23]. In contrast, aortic chondroitin sulfate+dermatan sulfate has approximately 100 sulfate groups/100 disaccharides, as the majority of these glycosaminoglycans obtained from other tissues. Non-sulfated or disulfated disaccharide units constitute only a minor proportion of the total disaccharides found on the aortic chondroitin sulfate+dermatan sulfate (<5% of the total disaccharide units). Overall, our results show that aortic heparan sulfate is a poorly sulfated glycosaminoglycan whereas chondroitin sulfate and dermatan sulfate showed the expected sulfation degree.

3.2. Dermatan sulfate is the preponderant anticoagulant glycosaminoglycan obtained from human aorta

Aortic chondroitin sulfate+dermatan sulfate but not the heparan sulfate fraction has a significant anticoagulant activity, as evaluated by APTT assay. Typical assays are shown in Fig. 2A and B. In order to double APTT, higher concentration of aortic heparan sulfate (107.27 ± 46.06, $n=6$) is required than chondroitin sulfate+dermatan sulfate fraction (29.08 ± 13.08, $n=13$), 3.7-fold more on a weight-to-weight basis (Fig. 2C).

We further tested the anticoagulant activity of the purified fractions of aortic glycosaminoglycans on assays of thrombin inhibition in the presence of plasma cofactor

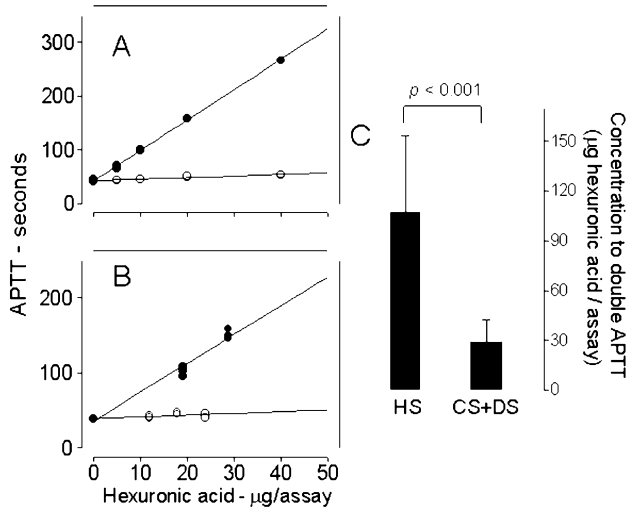

Fig. 2. Anticoagulant activity of purified aortic glycosaminoglycan fractions based on APTT assay. Panels A and B show typical experiments of clotting time versus concentrations of aortic glycosaminoglycans, using purified chondroitin sulfate+dermatan sulfate (●) and heparan sulfate (○) fractions obtained from normal thoracic aorta. The fittings in the panels were calculated as linear regression using a Microcal Origin PC program. Panel C shows the concentrations of aortic heparan sulfate (HS, mean ± SEM, $n=6$) and chondroitin sulfate+dermatan sulfate (CS+DS, mean ± SEM, $n=13$) necessary to double the APTT time. The difference between the two fractions was significant at the level of $p<0.001$ based on the Mann–Whitney U test.

(Fig. 3). Chondroitin sulfate+dermatan sulfate stimulates thrombin inhibition by heparin cofactor II (closed circles in Fig. 3A). After incubation with chondroitin AC lyase, which digests chondroitin sulfate but not dermatan sulfate, the thrombin inhibitory activity remains (open circles in Fig. 3A).[1] Digestion with chondroitin ABC lyase totally abolishes the activity (not shown). The thrombin activity is only marginally inhibited by the aortic heparan sulfate on assays performed in the presence of either heparin cofactor II (Fig. 3B) or antithrombin (Fig. 3C).[2] Similar results were obtained when the experiments were performed with the glycosaminoglycans extracted from human saphena veins (not shown). We did not attempt to correlate the anticoagulant activity of the vessel wall glycosaminoglycans with sex, age or some known cardiovascular risk factor such as high blood pressure, smoking, diabetes and lipoproteins concentrations. This type of study requires a larger number of affected patients. In addition, some of these clinical parameters were not available for the individuals included in this study.

[1] We would expect even an increase in the thrombin inhibitory activity of the purified dermatan sulfate when compared to the chondroitin sulfate+dermatan sulfate mixture on a weight-to-weight basis, which did not occur. Possibly the digestion with chondroitin AC lyase slightly decreased the molecular size of the dermatan sulfate and contributed to reduce its anticoagulant activity.

[2] Aortic heparan sulfate shows low anticoagulant activity regardless of the method used for its purification. Both the fraction purified by anion exchange chromatography and by digestion with chondroitin ABC lyase have low activity on APTT and thrombin inhibition assays.

In conclusion, our results indicate that dermatan sulfate is the preponderant anticoagulant glycosaminoglycan from human vessel walls.

3.3. Chondroitin sulfate+dermatan sulfate is the major antithrombotic glycosaminoglycan extracted from the arterial wall

Intravascular injection of aortic chondroitin sulfate+dermatan sulfate but not of heparan sulfate into normal rats, before the thrombotic challenge with thromboplastin, caused an inhibition of thrombus formation (Fig. 4). In these

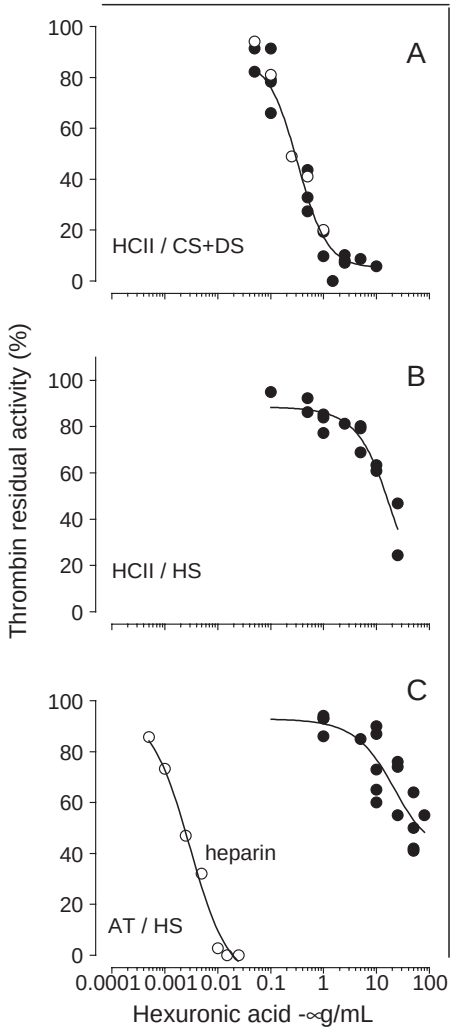

Fig. 3. Dependence on the concentration of aortic chondroitin sulfate+dermatan sulfate (A) or heparan sulfate (B and C) for inactivation of thrombin by heparin cofactor II (HCII, Panels A and B) or antithrombin (AT, Panel C). Heparin cofactor II (150 nM) or antithrombin (100 nM) were incubated with thrombin (20 nM) in the presence of various concentrations of the aortic glycosaminoglycan. After 60 s, the remaining thrombin activity was determined with a chromogenic substrate ($A_{405\ nm}$/min). The experiment of Panel A was performed with aortic chondroitin sulfate (CS)+dermatan sulfate (DS) fractions (●) and with a purified dermatan sulfate (○) preparation obtained by chondroitin AC lyase digestion. The fittings in the panels were calculated as a non-linear regression using a Microcal Origin PC program.

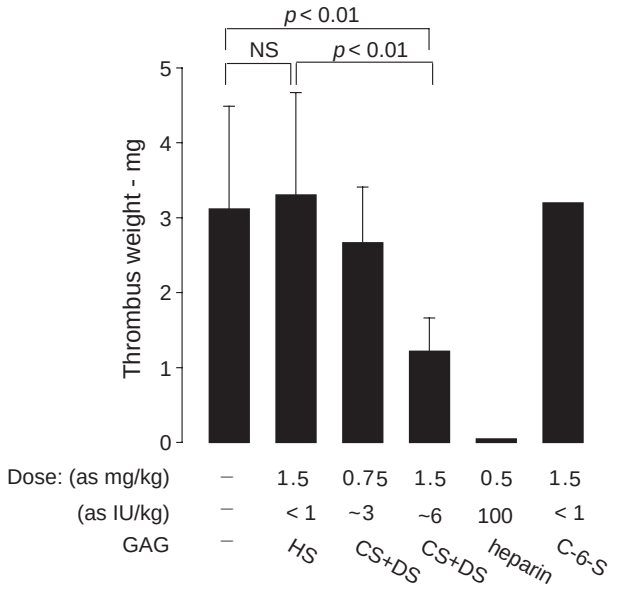

Fig. 4. Antithrombotic activity of aortic heparan sulfate (HS) and chondroitin sulfate+dermatan sulfate (CS+DS) fractions and standards of heparin and chondroitin 6-sulfate (C-6-S). Antithrombotic activity was investigated using a venous thrombosis model. Thrombus weight as mg (mean ± SEM) is expressed at various glycosaminoglycan doses (mg or IU/kg body weight). Results with significant difference ($p<0.01$) or not significant (*NS*) based on the Mann–Whitney test are shown in the Panel. C-6-S, standard chondroitin 6-sulfate.

experiments it was not possible to test the purified dermatan sulfate fraction due to the small amount of available material, but standard chondroitin 6-sulfate showed no activity up to the dose of 1.5 mg/kg body weight. These experiments indicate that the aortic chondroitin sulfate+dermatan sulfate mixture is antithrombotic (possibly due to the dermatan sulfate isomer) whereas the heparan sulfate fraction has no effect.

Higher doses of chondroitin sulfate+dermatan sulfate than that of heparin on weight basis are required to achieve the maximum effect. When the results are expressed as specific anticoagulant activity of the glycosaminoglycans (IU/Kg body weight), chondroitin sulfate+dermatan sulfate is more effective than heparin. Thus the antithrombotic action of dermatan sulfate is achieve with a significant lower effect on the blood coagulation than required for heparin. Similar observation has been reported previously for dermatan sulfate from other source [24]. Possibly, this event may be related with the fact that surface-bound thrombin is resistant to heparin/antithrombin but not to dermatan sulfate/heparin cofactor II inhibition, as reported [25,26]. Therefore, dermatan sulfate effectively decreases vessel wall thrombogenicity at a lower anticoagulant dose than heparin.

3.4. A minor fraction of the aortic heparan sulfate binds to antithrombin

The experiments of Figs. 2–4 were performed with the glycosaminoglycans extracted from the media+intima layers of the arterial or venous wall. However, under physiological conditions, the contact between the vessel wall and plasma occurs exclusively through the thin endothelial layer. Of course, heparan sulfate from this endothelial layer is only a minor component of the overall glycosaminoglycan extracted from the vessel wall. It is conceivable that endothelial cells may synthesize a particular heparan sulfate with high anticoagulant activity, which is not detected in the overall mixture of arterial wall

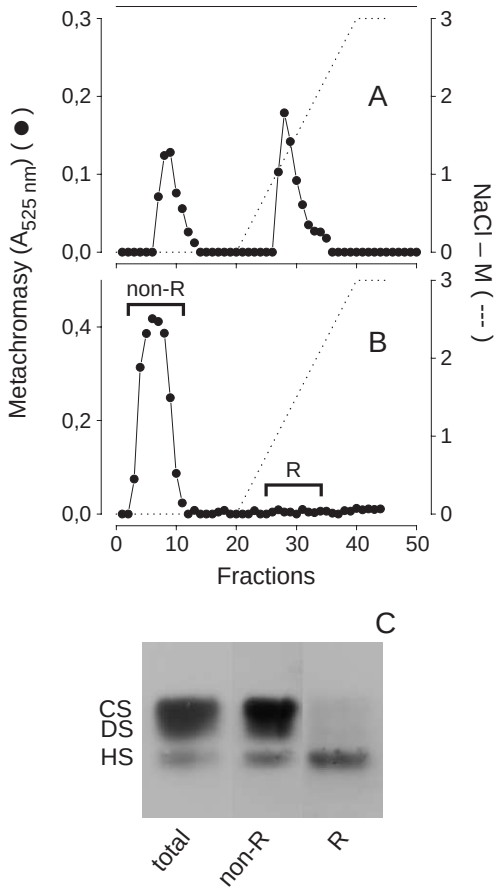

Fig. 5. Antithrombin affinity chromatography of glycosaminoglycans extracted from thoracic aorta. Standard heparin from porcine intestinal mucosa (200 μg, A) and glycosaminoglycans extracted from thoracic aorta (4 mg, B) were applied to an antithrombin affinity column (HiTrap NHS-activated 1 mL, coupled with 10 mg antithrombin), connected to an FPLC system. The column was eluted with a linear gradient of 0.15→2.0 M NaCl at a flow rate of 0.5 mL/min. The fractions were checked by the metachromasy produced with 1,9-dimethylmethylene blue. Retained (R) and non-retained fractions (non-R) were each pooled, as indicated by the horizontal bars in panel B, precipitated with 3 volumes of ethanol and dissolved in distilled water. The retained and non-retained fractions were analyzed by agarose gel electrophoresis. For this experiment the glycosaminoglycans samples were applied to 0.5% agarose gel and electrophoresis was carried out in 0.05 M 1,3-diaminopropane : acetate (pH 9.0) for 1 h at 120 V. The glycosaminoglycans in the gel were fixed with 0.1% *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide for 12 h and stained with 0.1% toluidine blue in acetic acid : ethanol : water (0.1 : 5 : 5, v/v) (C). The electrophoretic mobility of standard chondroitin 4-sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS) are indicated at the left of the panels.

glycosaminoglycans. In order to clarify this aspect a large amount of aortic glycosaminoglycan (~4 mg) was applied to an antithrombin-affinity column (Fig. 5). Fig. 5A shows the elution of heparin from the column. As seen, there are two populations of heparin, one that is retained (eluting at ~1.0 M NaCl) and another one that is not retained in the column. The latter population corresponds to molecules of heparin devoid of the pentasaccharide sequence, which ensures high affinity for antithrombin [27,28]. In contrast, aortic glycosaminoglycans do not bind to the antithrombin-affinity column (Fig. 5B), even when the column is loaded with a large amount of glycosaminoglycan (~4 mg). In an attempt to identify minor amounts of a population of aortic heparan sulfate, which might be retained in the antithrombin affinity column, we highly concentrated the fractions eluted between fractions 25 and 32 (indicated by a horizontal bar in Fig. 5B) although the presence of glycosaminoglycan was not detected by the metachromasy assay. These fractions have the same elution time as the heparin population with high affinity for antithrombin. In fact, we were able to identify on an agarose gel electrophoresis minor amounts of aortic heparan sulfate retained on the antithrombin affinity column (<5% of the total aortic heparan sulfate) (Fig. 5C). Further structural characterization or test on anticoagulant assay of this heparan sulfate was not possibly due to the small amount of available material.

In conclusion, a minor fraction of aortic heparan sulfate is retained on an antithrombin column. This population is probably diluted in the glycosaminoglycan mixture extracted from the arterial or venous wall and constitutes only a minor proportion of the bulk heparan sulfate fraction.

4. Discussion

Glycosaminoglycans are found in the arterial and venous walls. The most abundant glycosaminoglycan in human aorta is chondroitin 4/6-sulfate (~2.2 μg as hexuronic acid/mg of dry, defatted tissue), which is 4–8-fold more abundant than in saphenous veins. The concentration of heparan sulfate is 2.5-fold higher in human aorta than in saphenous vein (~1.3 and ~0.4, respectively, as μg of hexuronic acid/mg of dry tissue). In contrast with these two glycosaminoglycans, dermatan sulfate is found in comparable concentrations in human arteries and veins (~0.97 and ~0.80 μg/mg of dry tissue, respectively) [14]. Dermatan sulfate is also found in comparable concentrations in human arteries with different susceptibility to atherosclerosis [15]. Furthermore, the amount of dermatan sulfate remains constant, whereas the concentration of chondroitin 4/6-sulfate increases, in arterial segments devoid of atherosclerotic lesions obtained from patients with increasing age [13].

In the present study glycosaminoglycans were extracted from the vessel wall, highly purified and their anticoagulant and antithrombotic activities tested on in vitro and in vivo assays, respectively. We demonstrated that dermatan sulfate is the major anticoagulant and antithrombotic polysaccharide among the arterial and venous wall glycosaminoglycans whereas heparan sulfate is only a minor component (<5%) of the total vessel glycosaminoglycans.

Possibly, under physiological conditions, the contact between the vessel wall and blood occurs exclusively through the endothelial cell layer. Under this circumstance, the heparan sulfate expressed by the endothelial cell surface, although a minor component of the vessel wall, interacts with antithrombin and dramatically enhances its rate of coagulation protease neutralization. Although only about 1% of the antithrombin present in the blood is bound to the endothelium [29], this tiny amount is critical for normal hemostasis, since a complete antithrombin deficiency is incompatible with human life [2,3] and a decrease in its expression by half is associated with an increased risk of venous thromboembolism [1].

Besides antithrombin, another pathway dependent on protein C serves to regulate thrombin activity at the endothelium surface. The protein C pathway is initiated when thrombin binds to thrombomodulin, a chondroitin sulfate-proteoglycan expressed by endothelial cells [30]. Once bound to thrombomodulin, thrombin undergoes a conformational change at its active site that converts it from a procoagulant enzyme into a potent activator of protein C. Activated protein C, in concert with its cofactor protein S, serves as an anticoagulant by inactivating activated factors V and VIII. The physiological importance of the protein C pathway is highlighted by the observation that mice totally deficient in thrombomodulin die in uterus [31].

Therefore, the occurrence of these two natural anticoagulant pathways, both expressed at the endothelial surface, is essential to tightly regulate thrombin activity and prevent excessive thrombosis.

Ours and other results [12,32,33] highlight the possible importance of a third natural anticoagulant pathway, expressed by the subendothelial layer of the vessel wall, enriched of dermatan sulfate-proteoglycans, and mediated by heparin cofactor II. This pathway is activated under pathological conditions, after endothelial cell injury. In fact, administration of dermatan sulfate significantly suppressed arterial thrombotic occlusion induced by endothelial cell injury [34,35]. This is particularly significant since smooth muscle cells from the vessel walls proliferate, as they are exposed to plasma, after endothelium injury and these cells synthesize high amounts of dermatan sulfate [36]. Furthermore, dermatan sulfate content is elevated in atherosclerotic plaque compared with normal aorta [37,38]. Finally, an immunohistochemical study indicated that heparin cofactor II is distributed throughout the intima beneath the endothelium of normal human arteries [39].

In view of the above proposition it is consistent that heparin cofactor II-deficient mice undergo normal life with no evidence of thrombosis [12]. These animals possess normal antithrombin/heparan sulfate and protein C pathways expressed at the endothelial cell surface. However, as

the animals are challenged with an endothelial cell lesion (and suppression of the anticoagulant mechanisms expressed by the vascular endothelium), they are more susceptible to arterial thrombosis than control animals, which may be prevented by heparin cofactor II infusion [12]. On the other hand, mice deficient in a 3-sulfated heparan sulfate, and therefore devoid of a major ability to activate antithrombin, have a normal life with no evidence of thrombosis [6]. Possibly, the integrity of the two other anticoagulant pathways in these animals, mediated by protein C at the endothelial surface and by heparin cofactor II/dermatan sulfate at the subendothelial layer, compensates for the reduction of the antithrombin/heparan sulfate pathway.

Besides the effects on hemostasis and thrombosis, thrombin also plays a critical role in several other cellular events involved in the response to vascular injury, such as inflammation, healing and the less advantageous processes of atherosclerosis and restenosis. Thrombin is a mitogen for vascular smooth muscle cells [40,41] and fibroblasts [42,43], a chemoattractant for monocytes [44,45], etc. Thrombin may induce smooth muscle cell proliferation both directly or by causing platelets to secrete platelet-derived growth factor [46]. It also cleaves G-protein-coupled protease activated receptors on the platelet membrane to stimulate platelet aggregation and degranulation [47]. In an injury model of rabbit carotid artery, acute infusion of dermatan sulfate prevented neo-intimal hyperplasia [48]. Furthermore, a reduced incidence of restenosis after percutaneous coronary intervention may be related with plasma heparin cofactor II activity [33], which inactivates thrombin in injured arteries, thereby inhibiting vascular smooth muscle cell migration and proliferation. Heparin cofactor II has also been proposed as an antiatherogenic factor and a stronger predictive factor than HDL cholesterol against carotid atherosclerosis in elderly individuals [49]. Finally, dermatan sulfate in atherosclerotic lesions exhibits altered structure and reduced heparin cofactor II activity, when compared with normal arteries [32]. Loss of the ability of dermatan sulfate in the atherosclerotic lesions to regulate thrombin activity through heparin cofactor II may be critical in the progression of the disease.

Taken together, these observations are consistent with the notion that the dermatan sulfate/heparin cofactor II pathway inactivates thrombin at the subendothelial layer of the vessel walls. This effect may prevent thrombosis on the initial stage of endothelium injury. Thrombin inactivation may have a further consequence, preventing proliferation of vascular smooth muscle cells and the consequent neo-intimal hyperplasia, and therefore the start and progression of atherosclerotic lesions.

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