Heparin/heparan sulfate anticoagulant glycosaminoglycans in human plasma of healthy donors: preliminary study on a small group of recruits

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Glycosaminoglycans in normal human plasma, mainly represented by chondroitin sulfates and heparan sulfates/heparin (HSGAGs), show a specific distribution in the Cohn–Oncley fractions of human plasma. In the present study we investigated their effects on coagulation. Plasma was fractionated following the procedure of Cohn–Oncley, and each fraction was treated for extraction of glycosaminoglycans after extensive proteolysis; the anticoagulant activity in the extracted samples was measured by activated partial thromboplastin time (APTT). The effects of the samples containing HSGAGs on factor II and factor X activities, before and after treatment with heparinase I, were also measured. The molecular weight of HSGAGs was determined by polyacrylamide gel-electrophoresis. Cryoprecipitate and fraction I, fraction II+III, and fraction IV-1 (the fractions containing HSGAGs) prolonged the APTT, whereas fractions IV-4 and V had no effect on the APTT. Fractions containing HSGAGs showed effects on factor II and factor X activities that were sensitive to heparinase I treatment. The molecular weight of HSGAGs recovered in cryoprecipitate and fraction I was 15–18 kDa; that of HSGAGs recovered in fraction IV-1 was 12.0 kDa. In conclusion, these results demonstrate that HSGAGs of different molecular weight, endowed with anticoagulant activity, circulate in normal human plasma in association with specific proteins involved in the regulation of hemostasis; and that endogenous HSGAGs play a role in maintaining the antithrombotic/hemostatic balance in normal human plasma. *Blood Coagul Fibrinolysis* 19:349–354 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Human tissues, including plasma, contain glycosaminoglycans, which are polymers of disaccharide units. Glycosaminoglycans found in human tissues differ in molecular structure and the degree of sulfation. Hyaluronic acid is composed of acetyl glucosamine and glucuronic acid, and it is not sulfated. Keratan sulfate is sulfated, and it is composed of acetyl glucosamine and galactose. The class of chondroitin sulfate glycosaminoglycans (CSGAGs) encompasses chondroitin sulfate A and chondroitin sulfate C, which are composed of acetyl galactosamine and glucuronic acid. Chondroitin sulfate A and chondroitin sulfate C show various degrees of sulfation in different tissues. Chondroitin sulfate B also belongs to the class of CSGAGs, and is composed of acetyl galactosamine just like chondroitin sulfate A and chondroitin sulfate C; the uronic acid, however, is not only one, and both glucuronic acid and iduronic acid are present in the polymer. The class of heparan sulfate/heparin glycosaminoglycans (HSGAGs) encompasses heparan sulfate and heparin. All HSGAGs are sulfated and they are composed of acetyl glucosamine and of a uronic acid that could be either glucuronic acid or iduronic acid. The iduronic acid/glucuronic acid ratio is higher for heparin in comparison with heparan sulfate. All glycosaminoglycans are covalently linked to a protein core as proteoglycans [1]. The glycosaminoglycans in tissues and cells can be found either free, or noncovalently associated with proteins and phospholipids [2]. In human plasma all glycosaminoglycans can be found [3], and CSGAGs and HSGAGs are the most abundant [4,5]. Both classes of glycosaminoglycans interact with plasma proteins; HSGAGs, however, show the tightest association with plasma proteins, and this characteristic renders their extraction and purification from plasma quite difficult [6,7].

Among plasma glycosaminoglycans, HSGAGs are of particular interest because they are endowed with peculiar biological activities. Indeed, HSGAGs, and heparin in particular, are involved in the control of hemostasis and also regulate angiogenesis [8]. As far as the hemostatic balance is concerned, it is believed that

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HSGAGs, or at least some of them, exert a physiological antithrombotic activity analogous to that observed with their pharmacological counterparts (i.e. high-molecular-weight and low-molecular-weight heparins) [9]. In particular, since HSGAGs are strongly associated with plasma proteins, it is not known whether this physiological association modifies their anticoagulant, antithrombotic activities.

We recently developed a method to identify and quantify glycosaminoglycans in human plasma even when they are still associated with peptides [5], and we have described the distribution of CSGAGs and HSGAGs in fractions obtained by fractionating plasma according to the method of Cohn–Oncley [10]. In the present study, we evaluated the anticoagulant activity of the extracts obtained by both cryoprecipitate and Cohn–Oncley fractions (containing glycosaminoglycans and peptides, still associated after proteolysis). The effects on the activities of the coagulation factors II and X of those extracts containing HSGAGs were also measured, before and after treatment with heparinase I. The present study clearly demonstrated that HSGAGs endowed with anticoagulant activity circulate in normal plasma and that their association with specific peptides can be involved in regulating the hemostatic balance.

Materials and methods

Materials

Plasma was prepared from samples of 200 ml citrated blood. In this study, three 200 ml samples, each obtained from a different healthy donor, were provided by the Centre for Blood Transfusion of the Military Pharmaceutical Institute (Centro Trasfusionale Militare, Istituto Farmaceutico Militare) of Firenze, Italy. The donors were military recruits undergoing standard army physical training consisting of running (2 km/day), marching (5 km/day) and free fitness activity. The standard HSGAGs used in this study were: unfractionated heparin (UHP) [average molecular weight, 12.9 kDa; sulfation degree: SO_3^-/COO^-, 2.15; activated partial thromboplastin time (APTT), 64 IU/mg], extracted from bovine intestinal mucosa, and fractions of heparin, termed low-molecular-weight heparin (LMWHP 2123/850; average molecular weight, 4.5 kDa; sulfation degree: SO_3^-/COO^-, 2.19; APTT, 37 IU/mg) – both provided by Opocrin (Modena, Italy). Chondroitin sulfate A, chymotrypsin (EC 3.4.21.1), trypsin (EC 3.4.21.4), collagenase (EC 3.4.24.3), pepsin (EC 3.4.23.1), chondroitinase ABC (EC 4.2.2.4) and heparinase I (EC 4.2.2.7), were from Sigma Chemical Co. (St Louis, Missouri, USA). Papain (EC 3.4.22.2) was from Calbiochem (La Jolla, California, USA). Titan III Zip Zone cellulose acetate sheets ($60 \times 76\,mm^2$) were purchased from Helena Laboratories (Beaumont, Texas, USA). Alcian Blue was from Fisher Scientific Co. (Pittsburg, Pennsylvania, USA). Dialysis membranes (cutoff point, 3.5 kDa) were from Spectrum (Breda, The Netherlands). Azur A was from BDH (Poole, UK). Polyacrylamide gel electrophoresis (PAGE) reagents and all common other reagents were from Sigma Chemical Co. The reagents for evaluation of the APTT (APTT lyophilized silica for ACL analyzers) were from Instrumentation Laboratory (Milan, Italy). Factor II-deficient plasma (0008466050) and factor X-deficient plasma (0008466350), both from Instrumentation Laboratory, were used to evaluate the effects of HSGAGs on factor II and factor X activities, following the instructions of the manufacturer using an ACL Advance coagulometer.

Plasma protein fractionation according to the Cohn–Oncley method

Plasma obtained from each healthy donor was frozen in a glass beaker at −20°C for 24 h. The plasma was then defrosted at 4°C for 24 h, and afterwards it was centrifuged at $15\,000 \times g$ for 10 min. We obtained a supernatant, termed cryosupernatant, and a precipitate, termed cryoprecipitate. We then followed the Cohn–Oncley fractionation procedure as previously described [11].

Glycosaminoglycan extraction from cryoprecipitate and Cohn-Oncley fractions

Cryoprecipitate and fraction I, fraction II+III, fraction IV-1, fraction IV-4, and fraction V were suspended in 0.05 mol/l Tris–HCl, pH 7.4, and were treated with proteases added sequentially as follows: papain, trypsin, chimotrypsin, collagenase and pepsin [5]. After proteolysis, the sample was centrifuged at $15\,000 \times g$ for 10 min, and the supernatant was treated with ethanol to precipitate glycosaminoglycans [5]. The precipitate was resuspended in distilled water, dialysed against distilled water, and lyophilized. The retentate was dissolved in distilled water and stored at −80 until use.

Quantization of glycosaminoglycans

The retentate extracted from both the cryoprecipitate and the Cohn–Oncley fractions of human plasma was analyzed on cellulose acetate electrophoresis at pH 5.0, following the modification of this procedure that allows migration of glycosaminoglycans even in the presence of peptides or proteins [5]. To evaluate HSGAGs in the fractions, each sample was analyzed on cellulose acetate electrophoresis after treatment with nitrous acid that selectively degrades HSGAGs, according to Cappelletti *et al.* [12]. The bands were analyzed by densitometry for quantitative analysis using dedicated software (Scion Image; Scion Co., Gaithersburg, Maryland, USA), before and after treatment with nitrous acid. The results concerning quantization of glycosaminoglycans in human plasma are presented in Table 1 as the mean ± SD ($n = 3$ individuals).

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Table 1 Distribution of glycosaminoglycans in Cohn–Oncley fractions of human plasma

	Total glycosaminoglycans (ng)	HSGAGs (%)	CSGAGs (%)
Cryoprecipitate	514 ± 93	52.2	47.8
Fraction I	489 ± 24	48.4	51.5
Fraction II+III	606 ± 92	5.9	94.1
Fraction IV-1	483 ± 38	30.5	69.0
Fraction IV-4	174 ± 8	0.0	100.0
Fraction V	157 ± 32	0.0	100.0

Indicated amounts of glycosaminoglycans are referred to 1 ml starting plasma sample. Data presented as the mean ± SD of three different electrophoretic runs for each fraction obtained from the three healthy donors. Cryoprecipitate and fractions I, II+III, IV-1, IV-4 and V, fractions obtained by the Cohn–Oncley fractionation procedure; CSGAGs, class of chondroitin sulfates; HSGAGs, class of heparan sulfate and heparin.

Measure of the anticoagulant activity

Anticoagulant activity was measured by the APTT-based one-stage clotting time assay. Normal pooled plasma was used as the substrate, and the clotting time was recorded after adding appropriate amounts of each retentate [13].

Effect on factor II and factor X activity

The retentate from both the cryoprecipitate and Cohn–Oncley fractions I, II+III, and IV-1 (i.e. those containing HSGAGS) was evaluated for its effect on both factor II and factor X activity, either before or after treatment with heparinase I. An aliquot of each retentate was added to normal control plasma and the tests were performed following the instructions of the manufacturer (Instrumental Laboratories); results are reported as the percentage variation of the factor activity.

Quality controls concerning the APTT and factor II and factor X activity determinations were carried out on a daily basis in a Quality Certified Laboratory for Clinical Analysis (Institute for Clinical Research Manfredo Fanfani, Firenze, Italy) before performing the reported determinations. Quality controls were performed according to the Internal Quality Control System of the Institute and according to the External Quality Assessment System provided by the Reference Centre for Safety and Quality of the Italian Ministry of Health. For each test, the maximum and confidence limits are provided, and the expected and calculated mean values are considered.

Enzymatic treatments

Treatment with heparinase I was carried out in order to selectively degrade HSGAGs. Treatment was performed adding 10 IU enzyme to 10 μg total glycosaminoglycans of the sample for 24 h at 37°C [14].

Treatment with chondroitinase ABC was carried out in order to selectively degrade CSGAGs. Treatment was performed by adding 0.1 U enzyme to 10 μg total glycosaminoglycans of the sample for 24 h at 37°C [15].

Polyacrylamide gel electrophoresis analysis

Samples treated with chondroitinase ABC were analyzed by PAGE, following the method previously described [16]. After electrophoresis, gels were stained with Azur A in order to evidence glycosaminoglycans. The molecular weight of HSGAGs extracted from human plasma was determined by comparing the migration of standard HSGAGs of different molecular weight.

Results

Cohn–Oncley fractionation of plasma yields a cryoprecipitate and five fractions termed fraction I, fraction II+III, fraction IV-1, fraction IV-4, and fraction V. Each extracted fraction (the retentate) contains glycosaminoglycans and peptides still associated after extensive proteolysis [5].

Table 1 presents the results of the densitometric qualitative and quantitative analysis of glycosaminoglycan bands separated by cellulose acetate electrophoresis of retentates from both cryoprecipitate and Cohn–Oncley fractions of the healthy plasma donors used in this study. In particular, CSGAGs were recovered from both cryoprecipitate and all Cohn–Oncley fractions, whereas plasma HSGAGs sensitive to nitrous acid were recovered only from cryoprecipitate, and fractions I, II+III and IV-1. The results presented in Table 1 refer to the means obtained from three healthy donors, and it is worth noting that qualitative and quantitative distribution of glycosaminoglycans in these plasma samples paralleled that previously described [5].

The retentate from both cryoprecipitate and each Cohn–Oncley fraction was then tested for anticoagulant activity by the APTT. As a control, the same test was performed using different amounts of standard HSGAGs: UHP (average molecular weight, 12.9 kDa; APTT, 164 IU/mg), and low-molecular-weight heparin (average molecular weight, 4.5 kDa; APTT, 37 IU/mg) were used as standard HSGAGs with different anticoagulant activity (Table 2). The aliquot of each retentate tested for the APTT was that containing an estimated amount of HSGAGs of approximately 150 or 900 ng, in order to compare the effects with those of standard heparin used (see Table 2). These results demonstrated that only the retentates from fractions containing HSGAGs (i.e. cryoprecipitate and fractions I, II+III and IV-1), showed anticoagulant activity. The retentates from fractions containing uniquely CSGAGs (fractions IV-4 and V) did not show anticoagulant activity. The specific activity of naturally occurring HSGAGs was, however, significantly lower than that of UHP. We therefore decided to measure the molecular weight of the HSGAGs recovered from the cryoprecipitate and Cohn–Oncley fractions. Each retentate was treated with chondroitinase ABC, in order to selectively degrade CSGAGs, and was then analyzed by PAGE (Fig. 1). HSGAGs in cryoprecipitate and fraction I

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Table 2 Activated partial thromboplastin time of different amounts of the standard class of heparan sulfate and heparin (HSGAG) and of the sample obtained from cryoprecipitate and Cohn–Oncley fractions of human plasma

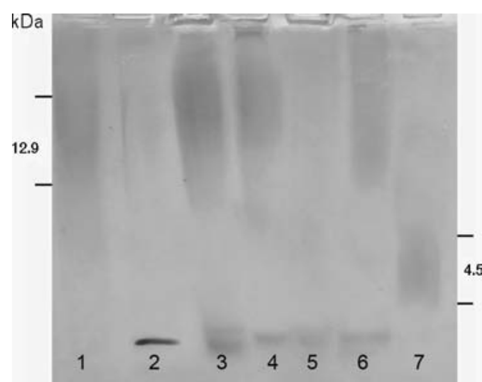
	Time (s)	Total glycosaminoglycans (ng)	HSGAGs (ng)
No addition	30.15		–
Unfractionated heparin	42.90	150	150
Unfractionated heparin	107.90	900	900
Low-molecular-weight heparin	35.30	150	150
Low-molecular-weight heparin	57.00	900	900
Cryoprecipitate	73.70	1676	900
Fraction I	72.70	1872	900
Fraction II+III	33.40	2070	150
Fraction IV-1	58.10	1783	900
Fraction IV-4	29.10	1659	0
Fraction V	28.35	1940	0

Results from a typical experiment representative of the three other different plasma fractionations that gave qualitatively identical results. The amounts of glycosaminoglycans (ng) represent those added to 500 μl pooled plasma used for the activated partial thromboplastin time. Unfractionated heparin activated partial thromboplastin time, 164 IU/mg; low-molecular-weight heparin activated partial thromboplastin time, 37 IU/mg. Cryoprecipitate and fractions I, II+III, IV-1, IV-4 and V, sample extracted from the corresponding fractions obtained by Cohn–Oncley fractionation procedure.

showed a high molecular weight (15–18 kDa); that is, higher than that of UHP (average molecular weight, 12.9 kDa). HSGAGs in fraction IV-1 showed a molecular weight of about 12.0 kDa, similar to that of UHP. PAGE could not evidence migration of chondroitinase ABC-resistant glycosaminoglycans in fraction II+III.

We then decided to measure the effects of the retentates containing HSGAGs and peptides on factor II and factor X activity, before and after treatment with heparinase I. The results are shown in Fig. 2. The amount of HSGAGs from each fraction, incubated for evaluating the effects on factor II and factor X activity, was the same as that of standard UHP (900 ng) used as a positive control, with the exception of fraction II+III (150 ng HSGAGs tested) (see legend to Fig. 2). According to the characteristics of the method, and to the quality control system adopted, variations less than 2 and 1% (for factor II and factor X activity, respectively) were considered in the normal range. Standard UHP showed an inhibitory effect sensitive to heparinase I on factor X activity (Fig. 2b, lane 2), whereas the inhibitory effect on factor II activity was close to the limit of the normal range; however, even this minimal inhibitory effect disappeared after heparinase I treatment (Fig. 2a, lane 2). The retentate from Cohn's fraction I showed an effect similar to that of UHP (Fig. 2a,b, lanes 4). Cryoprecipitate did not modify factor II and factor X activities, which remained within the normal range (Fig. 2a,b, lanes 3). Oddly enough, the treatment of cryoprecipitate with heparinase I evoked stimulation of factor II activity (Fig. 2a, lane 3). We interpret this result as the peptides in cryoprecipitate stimulating the activity of factor II, and this being inhibited by HSGAGs; heparinase I, removing this inhibition, restored the stimulatory effect. These two samples (i.e. retentates from cryoprecipitate and fraction I) were the most active in prolonging the APTT (i.e. the test measuring both the intrinsic and common pathways of coagulation system), so the results shown in Fig. 2 suggest that HSGAGs from these fractions are mostly inhibitory on the intrinsic pathway of coagulation, since evaluation of factor II and factor X activity were carried out using the prothrombin time test, which evaluates the extrinsic and common pathways of coagulation system.

Fig. 1



Polyacrylamide gel electrophoresis (PAGE) of heparan sulfates/heparin (HSGAGs) from Cohn–Oncley fractions of human plasma. Samples were run on PAGE and then colored with Azur A: HSGAGs showed evident methachromasia. 1, unfractionated heparin (average molecular weight, 12.9 kDa) (5 μg); 2, chondroitin sulfate A (5 μg), digested by chondroitinase ABC; 3, retentate from cryoprecipitate, digested by chondroitinase ABC; 4, retentate from fraction I, digested by chondroitinase ABC; 5, retentate from fraction II+III digested by chondroitinase ABC; 6, retentate from fraction IV-1 digested by chondroitinase ABC; 7, low-molecular-weight heparin (average molecular weight, 4.5 kDa) (5 μg). This was a typical experiment representative of three experiments, which gave qualitatively superimposable results.

The retentate from fraction II+III showed a stimulatory effect both on factor II and factor X activities (Fig. 2a,b, lanes 5). Treatment with heparinase I, however, changed the effect from stimulatory to inhibitory. The retentate obtained by fraction IV-1 showed a significant inhibitory effect both on factor II and factor X activity (Fig. 2a,b, lanes 6). The inhibitory effect on factor X activity was sensitive to heparinase I treatment in a manner similar to

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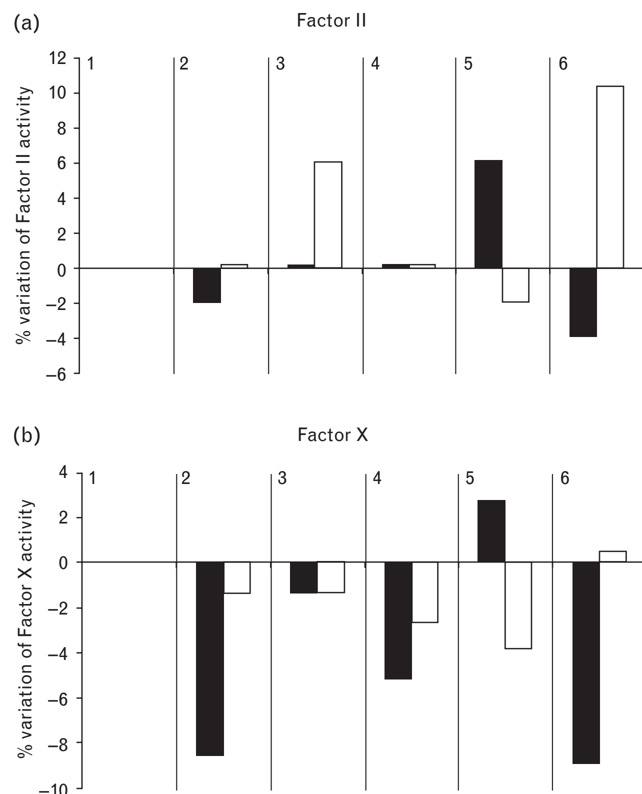
Fig. 2



Factor II and factor X activity. The activity of (a) factor II and (b) factor X was measured using a pooled plasma incubated with standard deficient plasma in the absence of (lane 1) or in the presence of 900 ng standard unfractionated heparin (lane 2). The effect of the retentate from both cryoprecipitate and Cohn–Oncley fractions was measured by adding 900 ng heparan sulfates/heparin (HSGAGs) from cryoprecipitate (lane 3), fraction I (lane 4) and fraction IV-1 (lane 6), and 150 ng HSGAGs from fraction II+III (lane 5) to pooled plasma. The effect was measured before (solid bars) and after (white bars) treatment with heparinase I. Reported are the results from one experiment representative of three others, which gave qualitatively identical results.

that observed with UHP or with the retentate from Cohn–Oncley's fraction I. In other words, treatment with heparinase I restored factor X activity within the normal range. As far as factor II activity was concerned, treatment of the retentate obtained by fraction IV-1 with heparinase I shifted the effect from inhibitory to stimulatory in analogy to what observed with cryoprecipitate (Fig. 2a, lane 3).

Discussion

Glycosaminoglycans play a role in different physiological and pathological conditions [1,8]. Among plasmatic glycosaminoglycans, HSGAGs are of particular interest because of their multifaceted biological activities. Moreover, HSGAGs are known to show different, and in some cases opposite, biological properties [17]. All plasmatic glycosaminoglycans circulate in association with a number of proteins. The association between HSGAGs and plasma proteins was the object of several studies, but this notwithstanding, little is known about the physiological role of this strong association [3,6]. Indeed, plasmatic glycosaminoglycans, in particular HSGAGs, are so strongly associated with proteins that complete purification of HSGAGs from healthy individuals is quite a long and difficult process, unsuitable for routine analysis of plasmatic HSGAGs [7]. We therefore opted for analyzing plasmatic glycosaminoglycans even in the presence of peptides remaining after proteolysis [5]. We observed that CSGAGs were recovered from all Cohn–Oncley fractions of human plasma and cryoprecipitate, whereas HSGAGs were recovered only from cryoprecipitate, fractions I and IV-1, and, in a smaller amount, from fraction II+III.

We then decided to evaluate the APTT activity in the extracts obtained from these fractions. The first report on the usefulness of the APTT for monitoring anticoagulant therapy appeared in 1962 [18]; ever since, the APTT has become the most popular laboratory test for monitoring full-dose heparin therapy. We observed that the anticoagulant activity, assessed as the APTT, paralleled the distribution of HSGAGs. Plasmatic HSGAGs were heterogeneous for molecular weight. High-molecular-weight HSGAGs were associated with cryoprecipitate and fraction I; that is, those fractions containing proteases involved in the coagulation cascade [19–21]. Fraction IV-1, containing antithrombin III [22], showed HSGAGs of smaller size (12.0 kDa). The presence of high amounts of peptides in the retentate from fraction II+III could be responsible for the lack of visualization of HSGAGs by PAGE (Fig. 1). This result appears at odds with the results reported in Tables 1 and 2, where only a small amount of HSGAGs endowed with anticoagulant activity could be detected in fraction II+III. Moreover, the results of PAGE indicated that the low specific activity of naturally occurring HSGAGs, lower than that of standard UHP, could not be directly related to their molecular weight.

Worth noting is that the bulk of plasma proteins was partitioned in fractions II+III, and V (i.e. those fractions containing immunoglobulins and albumin, respectively). HSGAGs endowed with anticoagulant activity therefore did not appear randomly associated with plasma proteins. Most coagulation factors and proteins involved in the control of hemostasis are concentrated in cryoprecipitate (fibrinogen, von Willebrand/factor VIII complex) and in fractions I (factor IX, factor X and prothrombin) and IV-1 (antithrombin III and protein C) [19–23]. These are the fractions where the highest amount of anticoagulant HSGAGs was recovered, thus suggesting a specific association of HSGAGs with proteins involved in hemostatic balance. We previously demonstrated that these HSGAGs were not '*bona fide*' purified because they were still associated with peptides remaining after proteolysis;

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in fraction II+III, the HSGAGs/protein ratio is the lowest for HSGAGs [5]. The co-presence of peptides remaining after proteolysis could be responsible for the low specific activity as well as for some effect observed on factor II and factor X activity. In fact, the retentate from fraction II+III, which contains the highest amount of peptides, showed a stimulating effect on both factor II and factor X activity (Fig. 2a,b, lanes 5); however, this effect was sensitive to heparinase I treatment of HSGAGs, suggesting that interaction between peptides and HSGAGs of different molecular weight could modulate their activity. The increase of factor II activity observed when retentates from both cryoprecipitate and fraction IV-1 were treated with heparinase I could be also related to the presence of peptides with stimulating effect on factor II activity that are inhibited by HSGAGs.

More than 100 proteins have been identified that bind exogenous HSGAGs, including proteins involved in hemostasis (inhibitors of coagulation as antithrombin III, but also coagulation factors), many growth factors, proteins involved in lipid metabolism and proteins of the extracellular matrix [24]. Whether these proteins in plasma interact with endogenous circulating HSGAGs *in vivo*, however, is still unknown.

In conclusion, the results described in this paper demonstrate that different classes of endogenous plasmatic HSGAGs endowed with anticoagulant activity circulate in normal human plasma possibly complexed/assembled with specific proteins that could be separated by Cohn–Oncley fractionation of plasma. Although the number of tested participants was limited ($n = 3$), the results concerning the amount of HSGAGs recovered in plasma are consistent with those reported in the literature [4–7]. The authors also suggest that their binding to specific plasma proteins (and/or peptides resulting from proteolysis) could play a role in the hemostatic balance. Further studies are needed, however, to assess complexing of naturally occurring HSGAGs with plasma proteins.

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