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HEMOSTATIC PROPERTIES AND SERUM LIPOPROTEIN BINDING OF A HEPARAN SULFATE PROTEOGLYCAN FROM BOVINE AORTA

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The biologic properties of two major proteoglycans of bovine aorta, heparan sulfate proteoglycan and chondroitin sulfate-dermatan sulfate proteoglycan were compared. The heparan sulfate proteoglycan was isolated either by elastase digestion or by 4.0 M guanidine hydrochloride extraction, of aorta tissue, fractionated by CsCl isopycnic centrifugation and purified by chondroitinase ABC treatment. The first method resulted in considerably greater yield (about 70% of the total heparan sulfate proteoglycan of the tissue) than the second procedure (12% of total). The chondroitin sulfate-dermatan sulfate proteoglycan was obtained by 4.0 M guanidine-HCl extraction of aorta tissue followed by CsCl isopycnic centrifugation. The chemical composition of both heparan sulfate proteoglycan preparations was similar. Unlike the chondroitin sulfate-dermatan sulfate proteoglycan, which eluted in the void volume of Sepharose CL-6B column, the heparan sulfate proteoglycan preparations were each resolved into a high molecular weight fraction ($k_{av} = 0.18$ and 0.13) and a low molecular weight fraction ($k_{av} = 0.47$ and 0.36). The heparan sulfate proteoglycan preparations exhibited significantly more potent anticoagulant and platelet aggregation inhibitory activities than the chondroitin sulfate-dermatan sulfate proteoglycan. The protein core of the proteoglycan molecules did not seem to be essential for their hemostatic properties. The complex forming ability of the heparan sulfate proteoglycan with serum low density lipoproteins (LDL) was much less than that of chondroitin sulfate-dermatan sulfate proteoglycan in the presence and absence of Ca^{2+}. Interaction between heparan sulfate proteoglycan and LDL was also much more sensitive to changes in the ionic strength of the medium than that of chondroitin sulfate-dermatan sulfate proteoglycan and the lipoprotein. Since the total sulfate content of both proteoglycans is almost similar, the smaller molecular size and hence the lower overall charge density of the heparan sulfate proteoglycan appears to be partly responsible for its low affinity for LDL. The differences in biologic properties of the two proteoglycans might have implications in the pathophysiology of cardiovascular diseases.

Glycosaminoglycans of the arterial wall are considered essential in maintaining the structural integrity of the vasculature. In addition, they are also known to possess several biologic properties, e.g., anticoagulant activity [1–3], platelet aggregation inhibitory activity [4,5], and serum lipoprotein binding ability [6–9]. Since sulfated glycosaminoglycan occur in tissues covalently bound to proteins as proteoglycans, a better understanding of the physiologic functions can be obtained by studying intact proteoglycans rather than their

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component glycosaminoglycan.

It is now established that there are at least two types of aorta proteoglycans: a chondroitin sulfate-dermatan sulfate proteoglycan [10–14] and a heparan sulfate proteoglycan [12,15]. Recently, we [5] reported on the biologic properties of the chondroitin sulfate-dermatan sulfate proteoglycan from bovine aorta, but no data are yet available on similar studies of the heparan sulfate proteoglycan. Since heparan sulfate is a major component of the arterial wall glycosaminoglycan [15], information on the biologic properties of heparan sulfate proteoglycans is vital in fully understanding its physiologic function and the role it plays in the pathophysiology of vascular disorders. However, unlike chondroitin sulfate-dermatan sulfate proteoglycan, it is difficult to extract aorta heparan sulfate proteoglycan by common dissociative solvents. Fibrous proteins of the tissue need to be solubilized prior to extraction of the bulk of this proteoglycan [15]. In the present study heparan sulfate proteoglycan was isolated from bovine aorta both by elastase digestion and guanidine hydrochloride extraction of the tissue. The purified proteoglycan preparations were studied for their biologic properties in comparison with a previously reported chondroitin sulfate-dermatan sulfate proteoglycan.

Materials and Methods

Materials

Bovine thoracic aortas were obtained from a local slaughterhouse within 15 min of death. Surrounding tissue was removed by dissection and the intima-media was dissected from each aorta and cut into small pieces. The aorta pieces were immediately transferred into 0.15 M NaCl maintained at 37°C and containing 0.5% penicillin-streptomycin and protease inhibitors and transported to the laboratory.

Chromatographically purified collagenase (Form III, *Clostridium histolyticum*) was obtained from Advance Biofactures Corporation (Lynbrook, NY) and high purity elastase (115 U/mg, Porcine Pancreatic) from Elastin Products Company, Inc. (Pacific, MO); guanidine hydrochloride and ethylenediaminetetraacetic acid (EDTA) were purchased from Matheson, Coleman and Bell Manufacturing Chemists (Norwood, OH) and technical grade CsCl from Kawecki Berylco Industries (New York, NY); benzamidine-HCl was obtained from Aldrich Chemical Company (Milwaukee, WI); ϵ-aminocaproic acid, *N*-ethylmaleimide, Russel's viper venom, kaolin, thrombin, antithrombin III, α-*N*-benzoylarginine ethyl ester (BAEE), adenosinediphosphate (ADP) and bovine achilles tendon collagen (Type I) came from Sigma Chemical Company (St. Louis, MO). Chondroitinases were obtained from Miles Laboratories (Elkhart, IN) and D_2O from J.T. Baker Chemical Company (Phillipsburg, NJ). Sepharose CL-6B was purchased from Pharmacia (Uppsala, Sweden). Samples of glycosaminoglycan standards were available in our laboratory from other studies.

Isolation of heparan sulfate

Heparan sulfate proteoglycan was isolated from bovine aorta by two different procedures.

Method 1. This method, which is a modification of the procedure of Radhakrishnamurthy et al. [15], is as follows: Finely minced intima-media (100 g) was extracted twice with 0.15 M NaCl containing penicillin-streptomycin and nonspecific protease inhibitors (0.1 M ϵ-aminocaproic acid, 0.005 M benzamidine-HCl, 0.01 M EDTA, 0.005 M *N*-ethylmaleimide) at 37°C for 24 h. The residue remaining after NaCl extraction was digested twice with collagenase (15 000 units) in 0.025 M Tris-0.01 M calcium acetate, pH 7.4, containing protease inhibitors (no EDTA) at 37°C for 24 h. The undigested residue was hydrolyzed twice by elastase (25 000 units) in presence of the above protease inhibitors in 0.2 M Tris, pH 8.8, at 37°C for 24 h. The supernatants from these two digests were combined and dialyzed against 0.15 M NaCl containing protease inhibitors at 4°C. The proteoglycan materials were precipitated from the dialysate by the addition of 2.5 volumes of ethanol and 1% (w/v) potassium acetate and recovered by centrifugation.

The elastase solubilized proteoglycans were purified as shown in Fig. 1. Crude proteoglycan material was dissolved in 4.0 M guanidine HCl and subjected to CsCl isopycnic centrifugation at 40 000 rpm for 20 h at 15°C in a vertical tube rotor (Beckman VTi 50; Beckman Instruments

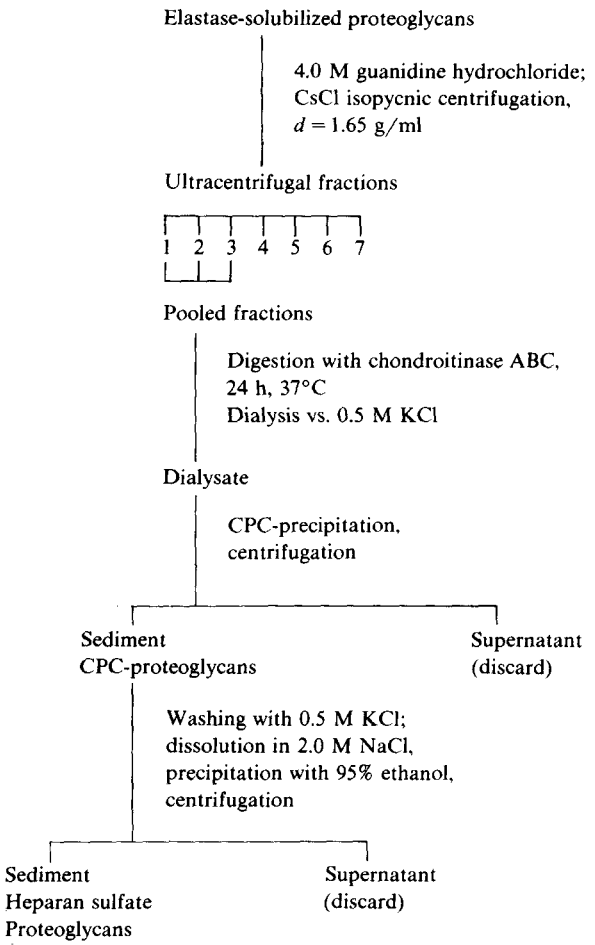

Fig. 1. Purification procedure of elastase-solubilized heparan sulfate proteoglycan from bovine aorta.

Inc., Palo Alto, CA) using a Beckman L5-75 ultracentrifuge. Following centrifugation, seven fractions (5 ml each) were collected. Bottom fractions 1–3 were pooled, dialyzed against 0.1 M Tris/0.1 M sodium acetate, pH 7.3, at 4°C and treated with chondroitinase ABC (1 U/ml) at 37°C for 24 h [the enzyme was checked for nonspecific protease activity by analyzing aliquots of the dialysate before and after the enzyme treatment for protein by the ninhydrin reaction [16]]. Following dialysis against 0.5 M KCl at 4°C for 24 h the proteoglycans were precipitated as their cetylpyridinium complexes by the addition of cetylpyridinium chloride to a final concentration of 0.5% [17]. After two washings with 0.5 M KCl the compex was dissolved in 2.0 M NaCl and the proteoglycan material was precipitated with 95% ethanol and dried in vacuo.

Method 2. In the second procedure heparan sulfate proteoglycan was isolated from bovine aorta intima-media (50 g) by 4.0 M guanidine hydrochloride extraction in the presence of protease inhibitors followed by CsCl isopycnic centrifugation essentially as reported previously for chondroitin sulfate-dermatan sulfate proteoglycan [18]. The seven proteoglycan fractions obtained after the second isopycnic centrifugation were dialyzed against distilled water at 4°C and lyophilized. Aliquots from each fraction were analyzed for glycosaminoglycan by cellulose acetate electrophoresis after β-elimination (see below) and the fractions (1–3) containing heparan sulfate were pooled (46.3 mg) and dissolved in 10 ml 0.1 M Tris-acetate buffer, pH 7.4. The proteoglycan solution was then treated with chondroitinase ABC (0.1 unit of enzyme/200 μg uronic acid) in the presence of protease inhibitors at 37°C for 8 h (the presence of nonspecific activity in the enzyme was checked as mentioned previously). The solution was then dialyzed against 0.5 M KCl at 4°C for 24 h and the heparan sulfate proteoglycan isolated from the solution as described in the first method.

Chondroitin sulfate-dermatan sulfate proteoglycan fractions were isolated and purified from bovine aorta intima after guanidine hydrochloride extraction as described before [18]. Chondroitin sulfate-dermatan sulfate proteoglycan fraction 4, whose biologic properties have been reported recently [5] was used from comparison of activities in the present studies.

β-Elimination of proteoglycan

β-elimination of the proteoglycan was carried out in 0.1 M NaOH for 24 h at 4°C as described before [18]. Following dialysis against distilled water, the glycosaminoglycans were eluted from a Dowex 50-X2 (H^+ form) column, neutralized with 0.1 N Na_2CO_3 and dialyzed against distilled water. Aliquots were subjected to gel filtration and cellulose acetate electrophoresis.

Electrophoresis

Electrophoresis of proteoglycans and their constituent glycosaminoglycans was performed on cellulose acetate plates along with appropriate glyco-

saminoglycan standards in pyridine-formic acid (pH 3.3) and 0.3 M cadmium acetate (pH 4.1) by previously described procedures [19,20]. In the case of heparan sulfate proteoglycan, electrophoresis was also conducted before and after chondroitinase ABC digestion and nitrous acid degradation of the proteoglycan.

Incubation of proteoglycans with collagenase and elastase

Proteoglycan (4.0 mg) dissolved in 4.0 ml of appropriate buffer containing protease inhibitors was incubated with collagenase (5 units) or elastase (5 units) at 37°C for 24 h. Following incubation, the tubes were heated at 80°C for 3 min in order to denature the enzyme. The contents of the tubes were then dialyzed overnight at 4°C against 0.5 M sodium acetate, pH 5.8. Control experiments were run without the additions of enzymes.

Gel filtration

Gel filtration of different proteoglycan fractions was performed on a Sepharose CL-6B column (1.5 × 100 cm) equilibrated with 0.5 M sodium acetate, pH 5.8 [21]. The void volume of the column was determined with *Escherichia coli* and the total volume with glucose. The elution volume of the proteoglycan was determined by analyzing fractions by an automated orcinol/H_2SO_4 reaction using a Technicon sugar chromatographic system. The column was run at a flow rate of 0.5 ml/min.

Isolation of low density lipoprotein

Low density lipoproteins (LDL, density, 1.02–1.063 g/ml) were isolated by sequentil ultracentrifugation of pooled human serum by the method of Hatch and Lees [22] using a type 40.3 rotor in a Beckman L5-75 ultracentrifuge. Solvent densities were adjusted using NaCl-NaBr solutions [23]. LDL was purified by recentrifugation at density 1.063 g/ml and was dialyzed against 0.15 M NaCl/0.01% EDTA, pH 7.0, at 4°C and stored under nitrogen in the cold. The purity of LDL was determined by immunodiffusion and by agarose gel electrophoresis [24,25]. The LDL was used for interaction studies within 3 weeks of its isolation.

Coagulation assays

Coagulation assays were done in a BBL Fibrometer (BBL, Cockeysville, MD). Blood for these assays was donated by laboratory personnel and collected over 0.1 volume of trisodium citrate (3.2%). Plasma was separated by centrifugation at 3500 cpm for 20 min at 4°C. Three clotting assays were performed [5]. The proteoglycans were dissolved in 0.14 M NaCl for all coagulation assays and platelet aggregation studies.

Strypven clotting time. A mixture of 0.1 ml of plasma, 0.1 ml of proteoglycan solution of different concentrations and 0.05 ml of Russel's viper venom was incubated for 2 min at 37°C. To the mixture 0.05 ml of 0.025 M $CaCl_2$ were added and the clotting time recorded.

Partial thromboplastin clotting time. To a mixture of 0.1 ml of plasma, 0.1 ml of proteoglycan solution (various concentrations) and 0.05 ml of kaolin (5 mg/ml) incubated for 3 min at 37°C, 0.05 ml of $CaCl_2$ (0.033 M) were added and clotting time recorded.

Thrombin time. A solution of 0.1 ml of thrombin (2 U/ml) was added to a mixture of 0.1 ml of plasma and 0.1 ml of proteoglycan solution (different concentrations) and the clotting time was recorded.

Effect of proteoglycans on thrombin-antithrombin III reaction

Experiments to determine the effect of aorta proteoglycans on thrombin-antithrombin III reaction were performed by measuring residual thrombin activity using α-*N*-benzoylarginine ethyl ester (BAEE) as a substrate [26]. These studies were conducted with only one heparan sulfate preparation (Method 1). A mixture of 2.4 ml of 0.15 M NaCl, 0.1 ml of thrombin (100 U/ml) and 0.1 ml of proteoglycan solution (1.5 mg/ml) was brought to 37°C. A solution of 0.1 ml antithrombin III (50 U/ml) was then added and the contents rapidly mixed. After incubation at 37°C for varying time intervals (1–5 min), 0.5 ml of BAEE (1 mg/ml in distilled water, warmed at 37°C) were added and the residual thrombin activity measured at 254 nm. Parallel experiments in which 0.15 M NaCl replaced proteoglycan and/or thrombin served as controls.

Platelet aggregation

The effect of proteoglycans on ADP-, collagen- and thrombin-induced platelet aggregation was studied as described before [5] in a platelet aggregation module (Model PAP-2, Bio/Data Corp., Norshaw, PA). Platelet-rich plasma and platelet-poor plasma were prepared from blood drawn from normal subjects. Platelet-rich plasma was preincubated for 1 min with proteoglycan solution of varying concentrations before the addition of aggregating agents. The light transmission registered 7 min after the addition of aggregating agent was considered as the end point of aggregation.

Proteoglycan-LDL interaction studies

The formation of insoluble complexes of proteoglycans was studied as described before [7]. Appropriate volumes of Tris buffer of varying molarities (0.001–0.1) and pH (5.0–7.4), various amounts of proteoglycan (25–300 μ/ml) and $CaCl_2$ (5–50 mM) were added to LDL (50–500 μg cholesterol/ml, final conc.) keeping the final volume to 2.0 ml. A reaction mixture with no proteoglycan served as the blank. The contents were mixed and after standing for 15 min at 37°C or room temperature, turbidity was measured at 600 nm against the blank. The insoluble complex was then separated by centrifugation, solubilized in 0.2 ml of 0.15 M NaCl and the amount of LDL in the complex was determined in terms of cholesterol.

The formation of soluble complexes of LDL and the proteoglycan was studied by ultracentrifugation at solvent density 1.063 g/ml [18]. LDL (1 mg cholesterol) was mixed with varying amounts of proteoglycan (200–800 μg) and the volume was brought to 2.25 ml with a buffer containing 0.14 M NaCl, 2.7 ml $CaCl_2$ or 0.14 M NaCl, 1 mM EDTA adjusted to pH 7.4 with 0.5 M Na_2HPO_4 [27]. Control experiments were made with proteoglycan alone. After standing at room temperature for 2 h, the solvent density was adjusted to 1.063 g/ml by adding 3.75 ml D_2O containing 30.66 mg NaCl (0.14 M) and then centrifuged under conditions used to isoate LDL. Following centrifugation, 6 fractions (1 ml each) were collected from the bottom to top of the tube and were analyzed for cholesterol and uronic acid as a measure of LDL and proteoglycan, respectively.

Analytical Methods

Total uronic acid was determined by the method of Dische [28], hexosamine by the method of Boas [29], cholesterol by the method of Pearson et al. [30] and protein by the method of Hartree [31]. Differential determinations of glucuronic acid and iduronic acid and glucosamine and galactosamine were performed by gas-liquid chromatography [32,33]. Total sulfate was determined by the rhodizonate method [34] after the sample had been hydrolyzed by 1.0 M HCl at 100°C for 3 h. N-sulfate groups in heparan sulfate proteoglycan were determined after nitrous acid degradation by the procedure of Lagunoff and Warren [35]. Hydroxyproline was determined by the method of Woessner [36] after hydrolysis of the sample with 6 M HCl for 3 h at 130°C.

Results

Isolation and chemical characterization of heparan sulfate proteoglycan

The CsCl isopycnic centrifugation profile of the eastase-solubilized proteoglycans (Method 1) is shown in Fig. 2. The proteoglycan fractions had different buoyant densities and uronic acid content. On cellulose acetate electrophoresis in cadmium acetate buffer fractions 1–3 gave two

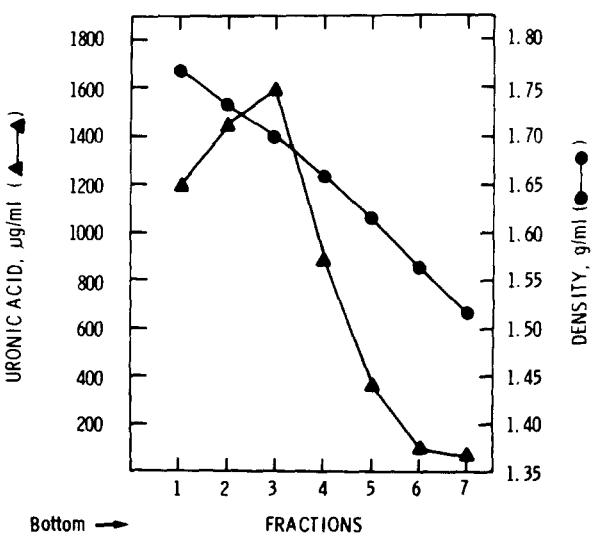

Fig. 2. Isopycnic CsCl centrifugation of elastase-solubilized proteoglycans from bovine aorta. Centrifugation was performed in a dissociative gradient at a loading density of 1.65 g/ml as described under Methods.

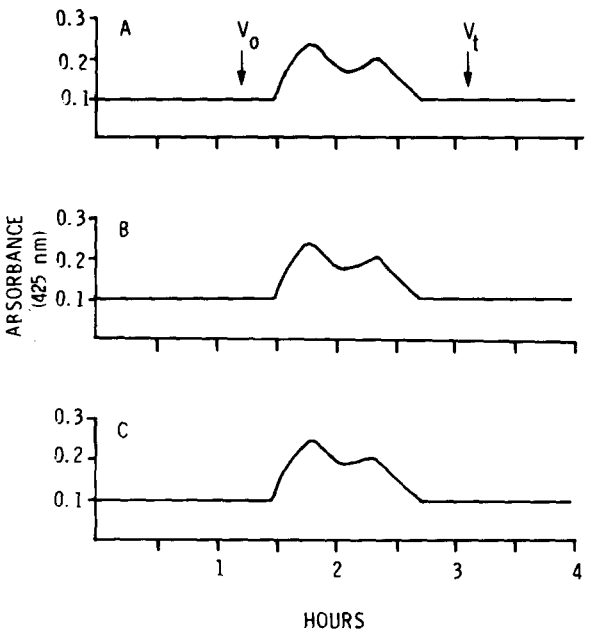

Fig. 3. Gel filtration of heparan sulfate proteoglycan isolated by method 1 on Sepharose CL-6B column before (panel A) and after digestion with collagenase (panel B) or elastase (panel C). The proteoglycan was eluted with 0.5 M sodium acetate, pH 5.8, at a flow rate of 0.5 ml/min.

minor fractions with mobilities corresponding to standard hyaluronic acid and chondroitin sulfate and a major fraction with a mobiity similar to heparan sulfate standard. Fractions 4 and 5 showed the presence of hyaluronic acid while fractions 6 and 7 showed no alcian blue positive materials. Based on electrophoretic mobilities and density gradient profiles, fractions 1–3 were pooled and subjected to further purification procedures. Treatment of the pooled fractions with chondroitinase ABC removed all traces of chondroitin sulfates. The enzyme did not hydrolyze the peptide bonds of the proteoglycan over a period of 48 h (μmol of leucine liberated at zero time, 24 and 48 h were: 3.3, 3.2 and 3.5, respectively), and therefore, the enzyme treatment for 24 h is unlikely to cause any cleavage of the protein core of the proteoglycans. Precipitation of the proteoglycans in the next step by cetylpyridinium chloride at a KCl concentration of 0.5 M resulted in the removal of all hyaluronic acid as evidenced by cellulose acetate electrophoresis. The purified heparan sulfate proteoglycan was resistant to degradation by collagenase and elastase in the presence of protease inhibitors as evidenced by identical elution profile of the proteoglycan before and after treatment with the enzymes (Fig. 3, panels B and C, respectively). In contrast, the chondroitin sulfate-dermatan sulfate proteoglycan was degraded by elastase, resulting in a shift of the material on Sepharose CL-6B column from excluded to included volume ($k_{av} = 0.43$). This indicates that the protease activity of elastase is not completely arrested even in the presence of inhibitors (heating the proteoglycan solution at 80°C for 3 min had no effect on their elution profile). The final yield of the heparan sulfate proteoglycan was 1.68 mg per g wet weight of the tissue, which is about 70% of the total heparan sulfate present in the aorta.

In the second procedure, the proteoglycan fractions 1–3 obtained after the second CsCl isopycnic centrifugation contained, in addition to heparan sulfate, dermatan sulfate, chondroitin sulfates and traces of hyaluronic acid. Heparan sulfate proteoglycan was isolated from the mixture by removing the dermatan sulfate and chondroitin sulfates by chondroitinase ABC treatment and hyaluronic acid by cetylpyridinium chloride precipitation of the proteoglycan in 0.5 M KCl. (Chondrointinase ABC was free of protease activity when incubated with the proteoglycan; micromoles of leucine liberated at zero time and 8 h were 2.9 and 2.7, respectively.) The yield of the proteoglycan was 0.29 mg per g wet weight of the tissue, which was 5.8-times less than the amount obtained by the first method. Therefore, only a few key studies of biologic properties were performed using this preparation.

The chemical composition of the purified heparan sulfate proteoglycan obtained by the two procedures is reported in Table I. The protein content of the two preparations was 16.2 and 18.6%, respectively. Approximately equimolar proportions of hexosamine and uronic acid were present in the proteoglycan. Glucosamine was the only hexosamine identified in both preparations, while they contained both glucuronic and iduronic acids in a proportion of 84 : 16 and 78 : 22, respectively. The hexosamine was partly N-sulfated (N-sulfate, 3.1 and 4.5%). The proteoglycan contained no hydroxyproline. Thus, the chemical composition of the two heparan sulfate preparations was very similar. Cellulose acetate electrophoresis of

TABLE I

CHEMICAL COMPOSITION OF HEPARAN SULFATE PROTEOGLYCAN OBTAINED FROM BOVINE AORTA BY TWO ISOLATION PROCEDURES

HS-PG 1 and HS-PG 2, heparan sulfate proteoglycan prepared by method 1 and method 2, respectively.

Chemical composition	HS-PG 1		HS-PG 2	
	% lyophilized weight	μM/mg	% lyophilized weight	μM/mg
Uronic acid	17.6	0.91	18.2	0.94
Glucuronic acid : iduronic acid [a]	84 : 16		78 : 22	
Hexosamine	18.9	0.88	19.3	0.90
Glucosamine : galactosamine [b]	100 : 0		100 : 0	
Protein	16.2		18.6	
Total sulfate	9.1	0.95	9.8	1.02
SO_4/hexosamine molar ratio	1.08		1.13	
N-Sulfate	3.1		4.5	
Glycosaminoglycan	100% HS		100% HS	

[a,b] Determined by gas-liquid chromatography [32,33].

the two purified proteoglycan preparations in cadmium acetate buffer showed one spot with mobility similar to heparan sulfate standard; however, electrophoresis following HNO_2 degradation of the proteoglycans and dialysis resulted in the complete disappearance of alcian blue positive materials. Furthermore, the proteoglycans were not digested by chondroitinase ABC. Based on these observations, the glycosaminoglycan in both proteoglycan preparations was characterized as heparan sulfate.

The chromatographic profile of the heparan sulfate proteoglycan (prepared by the first method) on Sepharose CL-6B column is shown in Fig. 3 (panel A). (The exlusion limit of polysaccharides for the column is $1 \cdot 10^6$.) The proteoglycan exhibited heterogeneity and resolved into a high molecular weight peak and a low molecuar weight peak with k_{av} values of 0.18 and 0.47, respectively. The relative proportion of the high and low moleclar weight fractions was 58.6 and 41.4%, respectively. However, cellulose acetate electrophoresis of the two fractions gave single spots with a mobility corresponding to standard heparan sulfate. The glycosaminoglycan chains released from this preparation of the heparan sulfate proteoglycan gave an included peak with a k_{av} of 0.63. Detailed studies of the two subpopulations of heparan sulfate proteoglycans were not attempted due to limitations of material. The elution profile of the heparan sulfate proteoglycan obtained by the second procedure, on Sepharose CL-6B column was similar to the first one. This preparation also gave two included peaks with k_{av} values of 0.13 and 0.36, respectively. The relative proportion of the two fractions was 52.8% and 47.2%, respectively.

The chemical composition of the current preparation of chondroitin sulfate-dermatan sulfate proteoglycan fraction 4 was essentially similar to the one reported previously [18]. It contained 23.2% uronic acid (glucuronate : iduronate, 90.0 : 10.0). 22.1% hexosamine, 17.9% protein and 10.2% total sulfate. The proteoglycan eluted in the void volume of Sepharose CL-6B column and, therefore, was much larger in size than the two heparan sulfate proteoglycan preparations.

Hemostatic properties

Anticoagulant activity. The anticoagulant activity of the two heparan sulfate proteoglycan preparations and chondroitin sulfate-dermatan sulfate proteoglycan preparation was compared in three clotting assays, viz, Stypven time, partial thromboplastin clotting time and thrombin time (Table II). Since the results obtained with both preparations of heparan sulfate proteoglycan were identical, the clotting times for the first preparation are only given in the table. Both types of proteoglycans delayed or totally inhibited clotting in all three

TABLE II

EFFECT OF PROTEOGLYCANS AND THEIR GLYCOSAMINOGLYCANS ON STYPVEN TIME, PARTIAL THROMBOPLASTIN CLOTTING TIME (PTT) AND THROMBIN TIME

Values are the mean of triplicate assays; coefficient of variation is given in parenthesis. HS-PG, heparan sulfate proteoglycan isolated by method 1; CS-DS-PG, chondroitin sulfate-dermatan sulfate proteoglycan; HS, heparan sulfate; CS-DS, chondroitin sulfate-dermatan sulfate. Clotting studies conducted with heparan sulfate proteoglycan isolated by method 2 gave results identical to the first preparation.

	Clotting time (seconds)		
	Stypven time	PTT	Thrombin time
Control	40.8 (3.3)	143.3 (3.6)	36.1 (1.7)
Proteoglycan (μg/ml):			
HS-PG:			
15.75	39.8 (2.9)	285.2 (2.1) ^{x,a}	98.5 (0.6) ^{x,a}
31.5	42.5 (1.9)	462.5 (2.2) ^{x,b}	No clotting
63.0	78.9 (1.2) ^{x,a}	763.4 (2.1) ^{x,c}	No clotting
94.5	89.5 (0.8) ^{x,b}	No clotting	No clotting
126.0	160.8 (1.2) ^{x,c}	No clotting	No clotting
378.0	450.4 (2.5) ^{x,d}	No clotting	No clotting
CS-DS-PG:			
16.0	38.9 (2.9)	156.8 (1.2) ^{y,a}	48.5 (1.6) ^{x,a}
32.0	41.8 (5.5)	262.6 (4.8) ^{x,b}	56.8 (4.0) ^{x}
64.0	63.5 (5.7) ^{x,a}	358.4 (2.0) ^{x,c}	376.6 (0.9) ^{x}
94.0	71.8 (4.2) ^{x,b}	575.8 (1.1)	795.5 (1.1) ^{x}
128.0	110.6 (2.1) ^{x,c}	685.5 (0.7)	No clotting
384.0	198.5 (1.6) ^{x,d}	948.8 (0.7)	No clotting
Glycosaminoglycan (μg uronic acid/ml)			
HS:			
10.0	73.5 (3.9) ^{x,e}	742.8 (1.8) ^{x,d}	No clotting
25.0	154.8 (6.1) ^{x,f}	No clotting	No clotting
CS-DS:			
10.0	53.2 (6.1) ^{x,e}	325.8 (1.6) ^{x,d}	362.8 (1.9) ^{x}
25.0	96.5 (4.2) ^{x,f}	620.5 (1.4) ^{x}	No clotting

^{x,y} Clotting times significantly differ from the corresponding control value at $P < 0.01$ and $P < 0.02$, respectively (Student's t-test).

$^{a-f}$ Clotting times with a common superscript within a column significantly differ at $P < 0.01$.

systems. The delay in clotting time was proportional to the concentration of the proteoglycan in the assay system. In all three systems the heparan sulfate proteoglycan was a more potent inhibitor of clotting than the chondroitin sulfate-dermatan sulfate proteoglycan: e.g., the Stypven time was delayed by 450 s by 378 μg/ml (66.5 μg uronic acid) of heparan sulfate proteoglycan, whereas 384 μg (89.0 μg uronic acid) of the chondroitin sulfate-dermatan sulfate proteoglycan delayed the clotting time only by about 200 s. Similarly, partial thromboplastin clotting time was completely inhibited by 94.4 μg/ml and thrombin time by 31.5 μg/ml of heparan sulfate proteoglycan, while similar results were achieved with the chondroitin sulfate-dermatan sulfate proteoglycan at a concentration above 384 μg/ml for partial thromboplastin clotting time and at 128 μg/ml for thrombin time. Coagulation assays were also performed using the free glycosaminoglycans obtained from the respective proteoglycans by β-elimination, in order to assess the effect of removal of the protein core of the proteoglycans on their anticoagulant activity. Thus, at approximatey equal amounts of uronic acid the prolongation of clotting time observed with each glucosaminoglycn was almost identical to that of its corresponding native proteoglycan (Table II). The difference in the anticoagulant activities of the two proteoglycans still persisted after removal of the protein core.

Effect of proteoglycans on thrombin-antithrombin III reaction

Studies were conducted to determine the effect of the two types of aorta proteoglycans on the inhibition of thrombin by antithrombin III. Figure 4 shows the inactivation of thrombin by antithrombin III in the presence and absence of proteoglycans as a function of time. The results are expressed as percent esterase activity of control. In the absence of any proteoglycan the inhibition of thrombin by antithrombin III was 28% at the end of 5 min, whereas in the presence of 100 μg of chondroitin sulfate-dermatan sulfate proteoglycan (23.2 μg uronic acid) or heparan proteoglycan (17.6 μg uronic acid) the inhibition of thrombin activity was 53 and 80%, respectively. Thus, the rate of reaction between thrombin and antithrombin III was accelerated more by the heparan sulfate proteoglycan than by the chondroitin sulfate-dermatan sulfate proteoglycan. Similar results were obtained from studies using free glycosaminoglycans isolated from the respective proteoglycans after alkali hydrolysis. Thus, in the presence of heparan sulfate equivalent to 20 μg uronic

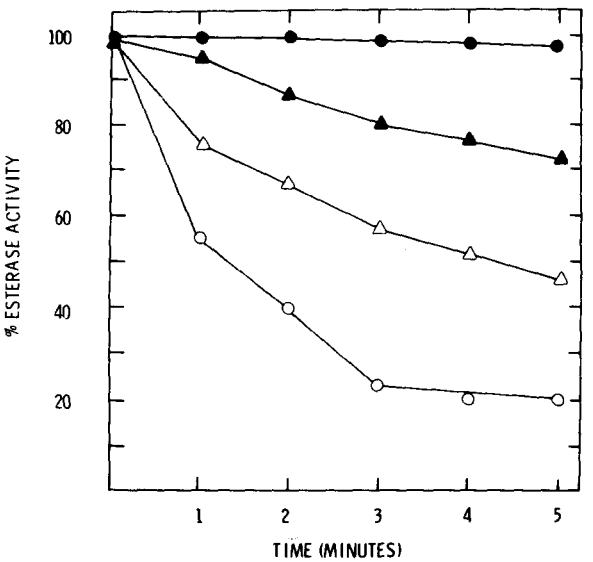

Fig. 4. Effect of proteoglycans from bovine aorta on thrombin-antithrombin III reaction. A solution of 0.1 ml of antithrombin III (50 U/ml) was added to a mixture of 2.4 ml of 0.15 M NaCl, 0.1 ml thrombin (100 U/ml) and 0.1 ml of proteoglycan solution (1.5 mg/ml). After incubation at 37°C for 1–5 minutes, 0.5 ml of BAEE (1 mg/ml) were added and the residual thrombin activity measured at 254 nm. Thrombin control (●———●) values remained constant throughout the incubation and represent 100% activity. The antithrombin III control (▲———▲) contained only thrombin and antithrombin III. chondroitin sulfate-dermatan sulfate proteoglycan (△———△); heparan sulfate proteoglycan (○———○) isolated by method 1.

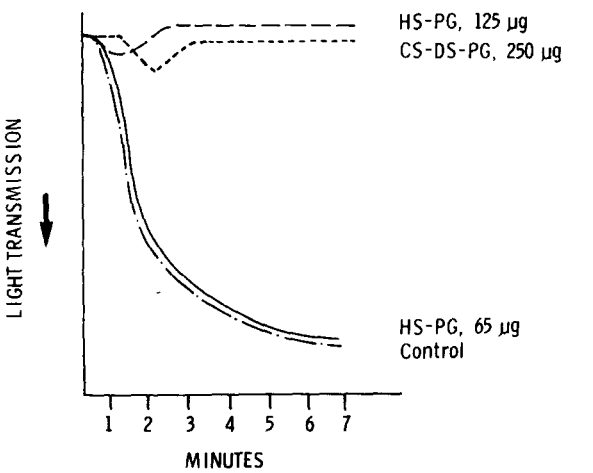

Fig. 5. Effect of bovine aorta proteoglycans on thrombin-induced platelet aggregation. Platelet aggregation was studied using normal platelet-rich plasma. Thrombin was added at a final concentration of 0.25 U/ml. CS-DS-PG, chondroitin sulfate-dermatan sulfate proteoglycan; HS-PG, heparan sulfate proteoglycan isolated by method 1; similar results were obtained with the second preparation.

acid, antithrombin III inhibited 82.5% of thrombin activity whereas in the presence of similar amounts of chondroitin sulfate-dermatan sulfate the inhibition of thrombin activity by the inhibitor was only 49%. These studies confirm the recent observations of Hatton et al. [26] using free glycosaminoglycans.

Platelet aggregation

Figure 5 shows the effect of proteoglycans on thrombin-induced platelet aggregation. At or above the concentration of 125 µg/ml (22.0 µg uronic acid) the heparan sulfate proteoglycan completely inhibited the aggregation induced by 0.25 U/ml (final concentration) of thrombin, while a two-fold higher concentration (250 µg/ml, 58.0 µg uronic acid) of chondroitin sulfate-dermatan sulfate proteoglycan was required in order to achieve the same effect. Thus, at comparable uronic acid levels the heparan sulfate proteoglycan was a more potent inhibitor of thrombin-induced platelet aggregation than the chondroitin sulfate-dermatan sulfate proteoglycan. However, at a concentration equivalent to 65 µg/ml the heparan sulfate proteoglycan did not inhibit thrombin-induced aggregation. Aggregation studies using heparan sulfate proteoglycan between the concentrations of 65–125 µg/ml were not carried out due to the lack of sufficient proteoglycan material. Similar studies conducted using free glycosaminoglycans from the respective proteoglycans gave identical results at equivalent uronic acid levels (data not shown). Neither proteoglycan affected platelet aggregation induced by ADP and collagen. Aggregation studies performed with the second preparation of heparan sulfate proteoglycan gave results identical to those obtained for the first preparation.

Proteoglycan-LDL interactions

Insoluble complexes Studies were conducted to determine the propensity of heparan sulfate proteoglycan in rendering serum LDL insoluble. These experiments were performed under a variety of reaction conditions by altering the pH and ionic strength of the medium as well as the concentrations of proteoglycans, LDL and Ca^{2+} as described under Methods. The heparan sulfate proteoglycan prepared by both procedures formed insoluble complexes with LDL only at or below

pH 6.0 and an ionic strength of 0.18. However, at that pH and in the presence of 30 mM Ca^{2+}, the amount of LDL converted into insoluble complex by 100 μg/ml of the two heparan sulfate proteoglycan preparations was 4.5 and 12.8%, respectively. On the other hand, as previously reported [18], the chondroitin sulfate-dermatan sulfate proteoglycan at 40 μg/ml and physiologic pH converted 98% of added LDL into insoluble complex in the presence of 30 mM Ca^{2+}.

Soluble complexes. Formation of soluble complexes of LDL and heparan sulfate proteoglycan (first preparation) was studied at physiologic pH and ionic strength in the presence and absence of 2.7 mM Ca^{2+}. The ultracentrifugal profiles of mixtures of proteoglycan (250 μg) and LDL (1 mg cholesterol) with and without Ca^{2+} at 1.063 solvent density is shown in Fig. 6. In the presence of Ca^{2+} all of the LDL and 15.4% of proteoglycan were recovered in the top fraction, whereas in the absence of Ca^{2+} only 4.5% of the proteoglycan was associated with the LDL in the top fraction. In similar studies using the second preparation of the heparan sulfate proteoglycan, the amount of proteoglycan recovered in the top fraction in the presence and absence of Ca^{2+} was 18.9 and 6.2%, respectively. In the absence of LDL all proteoglycan remained in the bottom fraction (not shown in figure). When similar studies were conducted with the chondroitin sulfate-dermatan sulfate proteoglycan the amount of the proteoglycan associated with LDL in the presence and absence of Ca^{2+} was 32.2 and 29.8%, respectively (data not shown).

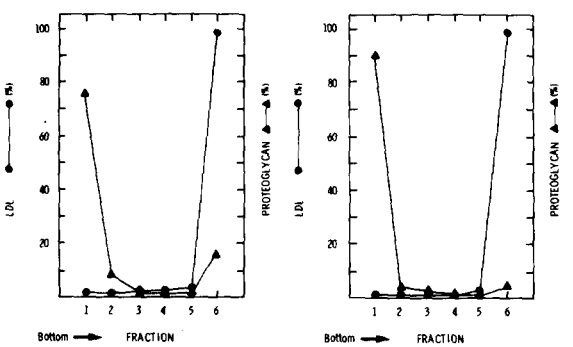

Fig. 6. Ultracentrifugal profile of LDL-heparan sulfate proteoglycan complexes with (left panel) and without (right panel) Ca^{2+}. Proteoglycan (250 μg) prepared by method 1 was mixed with LDL (1 mg cholesterol) in the presence and absence of 2.7 mM Ca^{2+} and the volume made up to 2.25 ml with 0.14 M NaCl, pH 7.4. After leaving the mixture at room temperature for 2 h, the solution density was adjusted to 1.063 g/ml by adding 3.75 ml of D_2O and 30.66 mg NaCl. The solutions were then subjected to ultracentrifugation at 114000× *g* for 20 h. Following centrifugation, 6 fractins (1.0 ml each) were collected from bottom to top and each fraction was analyzed for cholesterol and uronic acid as a measure of LDL and proteoglycan, respectively. Results are expressed as percent LDL and proteoglycan in each ultracentrifugal fraction. Similar observations were made with the heparan sulfate proteoglycan isolated by method 2.

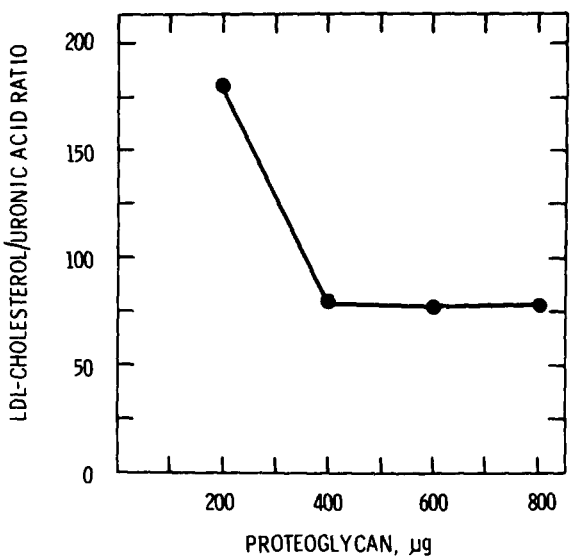

Fig. 7. Effect of heparan sulfate proteoglycan concentration on soluble complex formation with LDL. Varying amounts (200–800 μg) of proteoglycan were mixed with LDL (1 mg cholesterol) in the presence of 2.7 mM Ca^{2+} and subjected to ultracentrifugation as described in Fig. 6. Following ultracentrifugation, 6 fractions were collected and the amount of cholesterol and uronic acid in each fraction was determined. The proteoglycan binding to LDL is expressed in terms of cholesterol/uronic acid ratio in the top fraction. Studies conducted with the proteoglycan isolated by method 2 gave similar results.

Figure 7 shows the effect of heparan sulfate proteoglycan concentration (200–800 μg) on the formation of soluble complexes with LDL (1 mg cholesterol) in the presence of 2.7 mM Ca^{2+}. Following ultracentrifugation, the amounts of proteoglycan and LDL in the top fraction was expressed in terms of cholesterol/uronic acid weight ratios. The ratio of cholesterol to uronic acid decreased as the amount of proteoglycan in the system was increased up to 400 μg. At this level 18.2% of the added proteoglycan was contained in the soluble

complex. The cholesterol to uronic acid ratio of the complex remained constant at proteoglycan concentrations above 400 μg (177.7 μg/ml). Similar results were obtained when these studies were repeated using the second preparation of the proteoglycan. In that case, 21.2% of the proteoglycan was recovered in the soluble complex when the proteoglycan content of the system was 400 μg. In contrast, under similar conditions the soluble complex formed with chondroitin sulfate-dermatan sulfate proteoglycan contained 40% of the proteoglycan [18].

Binding of heparan sulfate proteoglycan and chondroitin sulfate-dermatan sulfate proteoglycan to LDL was also studied at increasing molarities of NaCl (0.035–0.14) at pH 7.4 in the absence of Ca^{2+} in order to determine the affinity of these two proteoglycans for the lipoprotein. Following ultracentrifugation of the proteoglycan-LDL mixture (proteoglycan equivalent to 70 μg uronic acid and 1 mg LDL-cholesterol) at solvent density 1.063, the cotent of proteoglycan in the top fraction was determined. The results are expressed in terms of the relationship of free/bound uronic acid ratios to NaCl concentration (Fig. 8). At all concentrations of NaCl a less amount of heparan proteoglycan was bound to LDL than chondroitin sulfate-dermatan sulfate proteoglycan. Thus, at 0.035 M NaCl the amount of each proteoglycan associated with the lipoprotein was 17 and 49%, respectively. There was a sharp linear increase with NaCl concentration in the free/bound uronic acid ratio for heparan sulfate proteoglycan with the result that at 0.14 M only 7% of the proteoglycan was associated with LDL. In contrast, the free/bound uronic acid ratio increased very little with NaCl concentration for the chondroitin sulfate-dermatan sulfate proteoglycan and 39% of the proteoglycan was bound to LDL at 0.14 M NaCl. These studies were not performed with the second preparation of the heparan sulfate proteoglycan due to nonavailability of material.

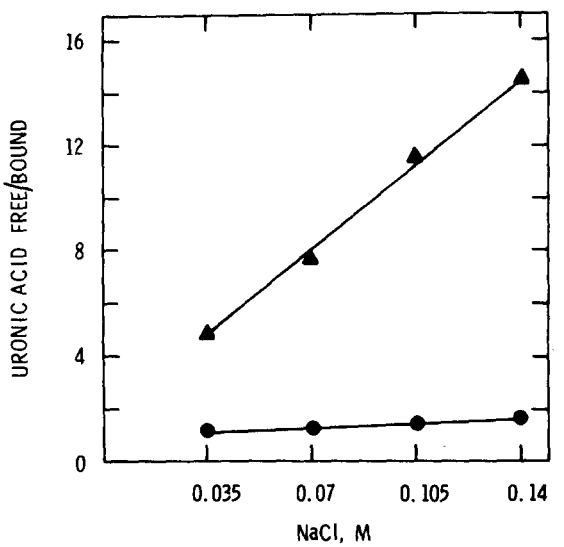

Fig. 8. Effect of ionic strength on proteoglycan-LDL interaction. Heparan sulfate proteoglycan isolated by method 1 and chondroitin sulfate-dermatan sulfate proteoglycan (both proteoglycans equivalent to 70 μg uronic acid) were separately mixed with LDL (1 mg cholesterol) and the volumes made up to 2.25 ml with NaCl solutions of varying molarities (0.035–0.14) at pH 7.4. The mixtures were then ultracentrifuged and fractions were collected as described in Fig. 6. The uronic acid and cholesterol content of the top fraction was determined. The results are expressed in terms of the relationship of free/bound uronic acid ratios to NaCl concentration. ▲——▲, heparan sulfate proteoglycan; ●——●, chondroitin sulfate-dermatan sulfate proteoglycan.

Discussion

Proteoglycans in the aorta are highly heterogeneous. While some of them are extractable by dissociative solvents such as 4.0 M guanidine hydrochloride, others require solubilization of fibrous proteins prior to their extraction. As indicated by the present studies and reported by other investigators [12], extraction of bovine aorta with 4.0 M guanidine hydrochloride releases only a small amount of the heparan sulfate proteoglycan. Since the majority of heparan sulfate proteoglycan in bovine aorta is associated with elastic fibers, digestion of the tissue by elastase is a feasible method for isolating the bulk of proteoglycan [15]. Therefore, in the present study aortic tissue was digested with elastase in presence of protease inhibitors in order to obtain a good yield of heparan sulfate proteoglycan. However, due to inherent protease activity of this enzyme, despite high purity, the possibility of some degradation of the native proteoglycan molecule during the isolation procedure does exist. Therefore, we also isolated this proteoglycan by extraction of aorta tissue by 4.0 M guanidine hydrochloride – a procedure used by several laboratories to isolate aorta chondroitin

sulfate-dermatan sulfate proteoglycan in the native state [12,14,18]. Coextracting chondroitin sulfates and dermatan sulfate are removed by digestion with chondroitinase ABC. Since chondroitinase ABC digestion of proteoglycans is used extensively to isolate their protein core [37–39] and no protease activity could be detected in the enzyme preparation in the current study, it is likely that the material obtained by this procedure might represent native heparan sulfate proteoglycan species. The high protein content and k_{av} values of heparan sulfate proteoglycan obtained by this method as compared to the one obtained by elastase digestion suggests that either elastase causes some degradation or the guanidine hydrochloride selectively extracts a high molecular weight fraction.

The gel filtration profile of the two heparan sulfate proteoglycan preparations indicates their heterogeneous nature which could be due to the existence in the aorta of two species of the proteoglycan, one of them occurring probably as elastin-bound, high molecular weight proteoglycan and the other as a low molecular weight form that exists as free proteoglycan. Two pools of heparan sulfate proteoglycans, one a lipid-bound, high molecular weight aggregate and the other a low molecular weight type that does not form micellar structures, have been isolated from rat liver plasma membranes by Kjellen et al. [40] and from cultured glial cells, endothelial cells and fibroblasts by Norling et al. [41]. More detailed compositional and structural studies will be required on the subspecies of aorta heparan proteoglycan to observe its characteristics in comparison to preparations that can be obtained from other tissues.

Even though the hemostatic properties of arterial glycosaminoglycans have been known for many years, it was demonstrated only recently that most of the activities were due to dermatan sulfate and heparan sulfate [2]. The reason for the difference in the anticoagulant activity of the two aorta proteoglycans revealed in the present study might be found in the difference in their respective structures. Heparan sulfate, which closely resembles heparin in its structure, might contain regions of the octasaccharide unit, IdUa-GlcNAC-GlcUA-$GlcNSO_3$-$IdUASO_3$-$GlcNSO_3$-$IdUASO_3$-$GlcNSO_3$, essential for the anticoagulant activity of heparin [42,43]. The increased inactivation of thrombin by antithrombin III in the presence of heparan sulfate proteoglycan (Fig. 4) lends support to this possibility. The N-sulfated glucosamine units of the proteoglycan might also contribute to its anticoagulant activity analogous to heparin [43]. The anticoagulant activity of chondroitin sulfate-dermatan sulfate proteoglycan, on the other hand, might be due to its dermatan sulfate content. However, the mode of action of dermatan sulfate in delaying clotting time is not known at the present time.

The antithrombin activity of the aorta proteoglycans appears to be due to the ability of polysaccharides to accelerate the inactivation of thrombin by antithrombin III (Fig. 4). Proteoglycans might do this by increasing the binding affinity of thrombin for antithrombin III as has been suggested for free glycosaminoglycans [26]. The proteoglycans also seem to potentiate the inactivation of other serine proteases involved in coagulation by antithrombin III, thereby prolonging the Stypven time and partial thromboplastin clotting time. It is also obvious from the present study that the heparan sulfate proteoglycan is a more potent inhibitor of thrombin-induced platelet aggregation than the chondroitin sulfate-dermatan sulfate proteoglycan. Thrombin-induced platelet aggregation is initiated by the interaction of thrombin with fibrinogen [44]. The heparan sulfate proteoglycan might inhibit this interaction more effectively than the chondroitin sulfate-dermatan sulfate proteoglycan. Furthermore, the glycosaminoglycan characteristics of the macromolecules but not the protein core appear to be responsible for the hemostatic properties.

Numerous studies have now shown complex formation between sulfated glycosaminoglycans and serum lipoproteins [6–9,27,45]. We have recently studied the interaction of serum lipoproteins and a chondroitin sulfate-dermatan sulfate proteoglycan from bovine aorta and showed that the protein core of the proteoglycan is essential for interaction with LDL [18]. The present studies were undertaken to investigate the interaction of the second major type of proteoglycan, viz, heparan sulfate proteoglycan, of bovine aorta with serum LDL. The results indicate that, irrespective of the mode of isolation of heparan sulfate proteoglycan,

there are marked differences between that proteoglycan and the chondroitin sulfate-dermatan sulfate proteoglycan in their ability to form soluble and insoluble complexes with LDL. Earlier studies by us [7,8] and others [9,27,45,46] suggest that the complexing ability of different free glycosaminoglycan depends not only on their degree of sulfation but on other structural characteristics as well. However, the reason for the low affinity of heparan sulfate proteoglycan towards LDL is not readily apparent from the chemical composition. Since the heparan sulfate proteoglycan contains almost equal amounts of total sulfate as the chondroitin sulfate-dermatan sulfate proteoglycan, the differences in the complexing abilities cannot be attributed to their sulfate content alone. Also, the heparan sulfate proteoglycan does not react as avidly with LDL as the chondroitin sulfate-dermatan sulfate proteoglycan in spite of its higher iduronic acid content (16 and 22% vs 10%). This indicates that, contrary to the suggestion made by Iverius [9], the presence of iduronic acid in a proteoglycan or glycosaminoglycan does not ensure its strong interaction with serum LDL. However, the chondroitin sulfate-dermatan sulfate proteoglycan used in this study, due to its larger size, must possess higher net charge density than the heparan sulfate proteoglycan. This proteoglycan with its high negative charge density might envelope domains of positive charges on the LDL molecule and promote cooperativity in binding [9] more effectively than the heparan sulfate proteoglycan. The observation that the removal of the protein core of the chondroitin sulfate-dermatan sulfate proteoglycan almost completely eliminated complex formation with LDL [18] despite the presence of iduronic acid in the proteoglycan lends support to this view. Since the nature of the proteoglycan, especially its size, could affect the optimal charge density, it is reasonable to suggest that the low affinity of the heparan sulfate proteoglycan for LDL might be partly due to its smaller molecular size.

Our studies thus suggest that the arterial wall heparan sulfate proteoglycan with its potent anticoagulant and platelet aggregation inhibitory activities on the one hand, and with its inability to interact avidly with serum LDL on the other might be functionally important. This proteoglycan, synthesized predominantly by the endothelial cells [47], might also increase the cell surface negative electrostatic charge without affecting LDL binding and thereby selectively provide an antithrombogenic surface and offer protection to the endothelium from platelet deposition analogous to heparin [48,49]. The pathophysiological significance of our findings in terms of atherosclerotic vascular disease is apparent.

Acknowledgements

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