Identification of the core proteins in proteoglycans synthesized by vascular endothelial cells

Anders LINDBLOM,* Ingemar CARLSTEDT and Lars-Åke FRANSSON

Department of Physiological Chemistry 2, University of Lund, P.O. Box 94, S-221 00 Lund, Sweden

Proteoglycans, metabolically labelled with [^3H]leucine and $^{35}\text{SO}_4^{2-}$, were isolated from the spent media and from guanidinium chloride extracts of cultured human umbilical-vein endothelial cells by using isopycnic density-gradient centrifugation, gel filtration and ion-exchange h.p.l.c. The major proteoglycan species were subjected to SDS/polyacrylamide-gel electrophoresis before and after enzymic degradation of the polysaccharide chains. The cell extract contained mainly a heparan sulphate proteoglycan that has a buoyant density of 1.31 g/ml and a protein core with apparent molecular mass 300 kDa. The latter was heterogeneous and migrated as one major and one minor band. After reduction, the apparent molecular mass of the major band increased to approx. 350 kDa, indicating the presence of intrachain disulphide bonds. The proteoglycan binds to octyl-Sepharose and its polysaccharide chains are extensively degraded by heparan sulphate lyase. The proteoglycans of the medium contained 90 % of all the incorporated $^{35}\text{SO}_4^{2-}$. Here the predominant heparan sulphate proteoglycan was similar to that of the cell extract, but was more heterogeneous and contained an additional core protein with apparent molecular mass 210 kDa. Furthermore, two different chondroitin sulphate proteoglycans were found: one 200 kDa species with a high buoyant density (approx. 1.45 g/ml) and one 100 kDa species with low buoyant density (approx. 1.3 g/ml). Both these proteoglycans have a core protein of molecular mass approx. 47 kDa.

INTRODUCTION

Endothelial cells provide the lining of the vascular system and, forming a barrier between blood and tissue, are thought to serve many different functions. Among these are selective transfer of various substances between blood and tissue as well as control of haemostasis. A variety of molecules, such as membrane proteins, proteoglycans and prostaglandins, have been shown to take part in these processes. The presence of heparan sulphate chains on endothelial cells, both *in vivo* [1] and *in vitro* [2–4], is well documented, and heparan sulphate proteoglycans on the endothelial-cell surface may play a critical role in the antithrombotic activity of the vascular surface [5,6]. Binding of both thrombin [7] and antithrombin III [8] to endothelial-cell cultures has been shown to depend on the presence of heparan sulphate. The heparan sulphate chains could thus serve as surface-bound co-factors for the inactivation of thrombin by antithrombin III. Thrombomodulin provides an alternative anticoagulant mechanism [9–11]. This endothelial-cell membrane protein has the ability to bind thrombin, and the resulting complex is a potent activator of Protein C, which then inhibits the coagulation cascade. Other functions assigned to endothelial-cell proteoglycans include anchoring lipoprotein lipase in adipose-tissue capillaries [12,13] and binding fibroblast growth factor, possibly as a 'supply' for the high-affinity fibroblast-growth-factor receptor [14].

Marcum *et al.* [8] showed that cloned endothelial cells are able to synthesize heparan sulphate with antithrombin III-cofactor activity. However, the activity resided in a small fraction (less than 1 %) of the heparan sulphate

proteoglycans, and the question therefore arises whether functionally distinct heparan sulphate chains occur in different core proteins. Identification of individual core proteins is a prerequisite for resolving this issue. Also, analysis of core proteins would serve to assess the purity of a proteoglycan preparation, and for most biological studies this information must be available. In the present study, we devised a protocol for the isolation of highly purified radiolabelled proteoglycans synthesized by cultured endothelial cells. The macromolecules are characterized by their overall size, 'hydrophobicity', buoyant density, glycosaminoglycan composition and size of the respective core proteins.

EXPERIMENTAL

Materials

Human umbilical cords obtained immediately after delivery were placed in Hepes-buffered saline (0.14 M-NaCl/4 mM-KCl/11 mM-D-glucose/15 mM-Hepes/NaOH buffer, pH 7.4) containing 10 μg of gentamycin/ml, and stored at 4 °C until used. Pooled human serum was a gift from the Department of Clinical Chemistry, and serum from healthy donors was purchased from the Blood Bank, Malmö General Hospital, Malmö, Sweden. The pooled serum was centrifuged (4000 g for 20 min at 4 °C) and filtered through a 0.2 μm -pore-size sterile filter (Millipore). Fibronectin was prepared from human plasma by affinity chromatography on Affi-Gel 15 (Bio-Rad Laboratories) substituted with gelatin, essentially as described by Engvall & Ruoslahti [15]. Another batch of human plasma fibronectin was a

Abbreviations used: CSPG, chondroitin sulphate proteoglycan; HSPG, heparan sulphate proteoglycan; PAGE, polyacrylamide-gel electrophoresis; NEM, *N*-ethylmaleimide; DFP, di-isopropyl phosphorofluoridate.

* To whom correspondence should be addressed.

gift from KABI-Vitrum, Stockholm, Sweden. The following materials were obtained from the sources indicated: Medium 199 from Gibco; heparan sulphate lyase (EC 4.2.2.8), heparin lyase (EC 4.2.2.7) and chondroitin ABC lyase (EC 4.2.2.4) from Seikagaku Kogyo Co.; gelatin (type I), ovalbumin, Hepes and gentamycin from Sigma Chemical Co.; Mulgophene non-ionic detergent (tridecyloxypolyethyleneoxyethanol) from GAF Corp., Johanneshov, Sweden; penicillin, streptomycin, L-glutamine and Fungizone from Flow Laboratories; collagenase (CLS 1) from Worthington Corp.; antiserum (immunoglobulin fraction) against human Factor VIII-related antigen and fluorescein isothiocyanate conjugated pig anti-(rabbit immunoglobulin) antibodies from Dako-patts; Sepharose CL-4B, octyl-Sepharose and a Mono Q/HR5/5/column from Pharmacia Fine Chemicals; DE-52 and DE-53 DEAE-cellulose from Whatman; $\text{Na}_2^{35}\text{SO}_4$ (carrier-free, 5.8 Ci/mmol) and L-[4,5- ^3H]leucine (40–60 Ci/mmol) from Amersham International. Stock solutions (approx. 8 M) of guanidinium chloride (practical grade) were treated with activated charcoal before use. Plastic tissue-culture flasks (25 and 75 cm²) and plastic Petri dishes (35 mm) were products of Costar or Nunclon. All other reagents were of analytical grade unless otherwise specified.

Cell culture

Endothelial cells were prepared from human umbilical veins and cultivated according to the procedure of Jaffe *et al.* [16]. The umbilical cords were used within 48 h after delivery. The vein was cannulated at one end and rinsed with 150 ml of Hepes-buffered saline, filled with 10–15 ml of 0.1% collagenase in Hepes-buffered saline and clamped. The slightly distended cord was placed in a sterile beaker containing pre-warmed Hepes-buffered saline and incubated for 10 min at 37 °C. The cord was then rubbed gently, and the collagenase digest was collected into a conical centrifuge tube containing 10 ml of Medium 199 containing 20% (v/v) human serum by flushing another 30 ml of Hepes-buffered saline through the vein. The cells were recovered by centrifugation (1000 rev./min for 5 min) in a Sigma 3-1 bench-top centrifuge, resuspended in 5 ml of fresh medium and seeded on to 25 cm² plastic tissue-culture flasks coated with fibronectin. Coating was achieved by treating the flasks with 250 μl of a fibronectin solution (1 mg/ml in 8 M-urea/50 mM-Tris/HCl buffer, pH 7.5) and 2 ml of Hepes-buffered saline at 4 °C overnight. Before seeding the flasks were rinsed once with 5 ml of Hepes-buffered saline to remove urea. Cells were grown in a CO₂ incubator at 5% CO₂ in air at 37 °C with Medium 199 supplemented with 20% (v/v) human serum, 2 mM-L-glutamine, penicillin (100 i.u./ml), streptomycin (100 μg /ml) and Fungizone (10 μg /ml). Plating and spreading occurred within 2–3 h after seeding on fibronectin-coated dishes, and the primary cell cultures usually became confluent within 2–5 days, depending on the number of cells in the preparation. Contaminating blood cells were removed by washing the cultures a number of times with Hepes-buffered saline 2–3 h after seeding.

After confluence, cells were passaged (split ratio 1:3), with the use of 0.05% collagenase. The culture medium was changed every 2–3 days. As a check of the endothelial origin of the cultured cells, a typical preparation was assayed for Factor VIII-related antigen by using immunofluorescence as described by Jaffe *et al.* [17]. The cells

could be cultivated for up to five passages without apparent loss of viability. However, after about 4 weeks the morphology gradually changed towards atypical 'sprouting cells'. Therefore only first-passage cultures with typical 'cobblestone' morphology were used and within 3 weeks after primary seeding of the cells.

Metabolic labelling

Confluent cultures (first passage and less than 5 days after confluence) were washed twice with Hepes-buffered saline and incubated with growth medium (0.2 ml/cm²) containing either $^{35}\text{SO}_4^{2-}$ (50 μCi /ml) and L-[^3H]leucine (20 μCi /ml) or L-[^3H]leucine (20 μCi /ml) alone. Sulphate-depleted medium could not be used to increase incorporation of $^{35}\text{SO}_4^{2-}$, because the cells detached from the flasks. After incubation for 48 h, the spent medium was carefully decanted and the cell layer was washed several times with ice-cold Hepes-buffered saline.

Isolation of proteoglycans

The cell layers were extracted by gentle shaking in 0.2 ml/cm² of 6 M-guanidinium chloride/1% (v/v) Triton X-100/10 mM- Na_2EDTA /10 mM-NEM/1 mM-DFP/50 mM-sodium acetate buffer, pH 5.8, containing 5 μg of ovalbumin/ml at 4 °C overnight. The labelled proteoglycans from the medium were partially purified by ion-exchange chromatography on DE-53 DEAE-cellulose at 4 °C. The column (2 ml) was equilibrated with 6 M-urea/10 mM-NEM/0.1% (v/v) Triton X-100/0.2 M-sodium acetate buffer, pH 5.8, containing 5 μg of ovalbumin/ml. After being loaded with the sample, the column was washed with 5 $\times$ 1 ml of the equilibrating buffer, followed by 5 $\times$ 1 ml of 6 M-urea/0.1% (v/v) Triton X-100/10 mM-Tris/HCl buffer, pH 8.0, and 5 $\times$ 1 ml of 6 M-urea/0.1% (v/v) Triton X-100/0.5 M-sodium acetate buffer, pH 5.8. Proteoglycans were eluted with 5 $\times$ 1 ml of 6 M-guanidinium chloride/1% (v/v) Triton X-100/10 mM- Na_2EDTA /10 mM-NEM/50 mM-sodium acetate buffer, pH 5.8, containing 5 μg of ovalbumin/ml.

CsCl and water were added to the cell extracts and the medium proteoglycans to an initial density of 1.31 g/ml in 4 M-guanidinium chloride, and the samples were centrifuged at 36000 rev./min for 70–80 h at 15 °C in a Beckman L8 60M centrifuge fitted with a 50.2 Ti rotor. After centrifugation, the tubes (40 ml) were emptied from the bottom and 2 ml fractions were collected.

Gel-permeation chromatography was performed on Sepharose CL-4B in 4 M-guanidinium chloride/0.2% (v/v) Triton X-100/50 mM-sodium acetate buffer, pH 5.8, containing 5 μg of ovalbumin/ml. The columns (2.5 cm $\times$ 100 cm or 1.0 cm $\times$ 100 cm) were eluted at 4 cm/h and sample volumes were adjusted to approx. 1% of the bed volume (5 ml and 0.5 ml respectively). Samples were dialysed against the elution buffer before chromatography.

Ion-exchange h.p.l.c. was performed on a Mono Q HR 5/5 column. The gradient was mixed at the low-pressure side with an LKB titanium-head pump connected to a model 2152 controller and a 2040-203 mixing valve. The system was fitted with a Pharmacia V-7 injector by using Teflon tubing, and Pharmacia pre-filters were placed both before and after the pump. The column was equilibrated with 7 M-urea/0.1% (v/v) Mulgophene/0.3 M-NaCl/10 mM-Tris/HCl buffer, pH 8.0, and 5–20 ml samples were applied. The flow rate was 0.5 ml/min and

0.5 ml fractions were collected. After being washed with starting buffer the column was eluted with a linear gradient of 0.3–1.2 M-NaCl in the buffer over 60 min. Before each run, the column was washed with 5 ml of 2 M-NaCl in 7 M-urea/0.1% (v/v) Mulgophene/10 mM-Tris/HCl buffer, pH 8.0. Pooled fractions were concentrated by chromatography on small columns (0.5 ml) of DE-53 DEAE-cellulose, after dilution (1:4) with 6 M-urea/10 mM-Tris/HCl buffer, pH 8.0. The column was equilibrated in 6 M-urea/0.1% (v/v) Triton X-100/10 mM-Tris/HCl buffer, pH 8.0, and, after being washed, eluted with 5 × 0.3 ml of 4 M-guanidinium chloride/0.2% (v/v) Triton X-100/50 mM-sodium acetate buffer, pH 5.8, containing 5 µg of ovalbumin/ml. The proteoglycans were recovered in 0.6 ml and stored at 4 °C.

Degradative methods

Digestions with heparan sulphate lyase (3 munits/ml) or heparin lyase (3 munits/ml) were performed in 3 mM-calcium acetate/0.1% (v/v) Triton X-100/10 mM-Na₂EDTA/10 mM-NEM/1 mM-DFP/10 mM-Hepes/NaOH buffer, pH 7.0, at 37 °C for 2 h. All samples contained 5 µg of ovalbumin/ml as a carrier and were dialysed against the digestion buffer before incubation. Alternatively, heparan sulphate side chains were degraded by deaminative cleavage by using the pH 1.5/HNO₂ method [18]. Digestions with chondroitin ABC lyase (50 munits/ml) were performed in 10 mM-Na₂EDTA/10 mM-NEM/1 mM-DFP/0.1% (v/v) Triton X-100/10 mM-Tris/acetate buffer, pH 7.0, at 37 °C for 2 h.

Reduction of disulphide bonds was performed in 4 M-guanidinium chloride/10 mM-dithiothreitol/50 mM-sodium acetate buffer, pH 5.8, at 37 °C for 5 h. Alkylation was subsequently achieved by adding a molar excess of iodoacetamide over dithiothreitol and keeping the solution in the dark overnight.

Polysaccharide chains were liberated by treating purified proteoglycans with 0.5 M-NaOH at room temperature overnight. The chains were recovered by stepwise elution from DEAE-cellulose (see above) after neutralization with 100% acetic acid and diluting the samples (1:1) with water.

Hydrophobic chromatography

Purified proteoglycans were dialysed against 6 M-urea/0.2 M-sodium acetate buffer, pH 5.8, and applied to DEAE-cellulose columns equilibrated with the same buffer. The column was then washed with 5 × 1 ml of this buffer, followed by 5 × 1 ml of 6 M-urea/2% (v/v) n-octyltetraoxyethylene/0.4 M-sodium acetate buffer, pH 5.8, to wash off residual Triton X-100. Proteoglycans were displaced with 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8, containing 5 µg of ovalbumin/ml, dialysed against the same buffer, mixed with 1 ml of octyl-Sepharose in small plastic columns (Bio-Rad Laboratories) and put on a shaker overnight at 4 °C. The octyl-Sepharose columns were eluted with 5 × 1 ml of 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8, containing 5 µg of ovalbumin/ml, followed by 5 × 1 ml of 1% (v/v) Triton X-100 in the same buffer.

Electrophoresis

SDS/PAGE was performed on gradient gels (3–8% or 3–12% with 3% stacking gels; T/C = 30/0.8), with the Laemmli buffer system [19]. Before electrophoresis,

samples were precipitated with 9 vol. of 95% (v/v) ethanol, redissolved in 50 µl of 4 mM-Na₂EDTA/5% (w/v) SDS/20% (v/v) glycerol/0.04% Bromophenol Blue/125 mM-Tris/HCl buffer, pH 6.8, and boiled for 3 min. Recovery of material after precipitation was over 90%, as estimated by radioactivity measurements. For reduction of disulphide bonds, 2-mercaptoethanol was added to a concentration of 10% (v/v). The gels were stained with 0.25% Coomassie Blue R-250, soaked in 1.3 M-sodium salicylate/5 mM-sodium phosphate buffer, pH 7.0, dried and exposed to Kodak XAR films for 1–10 days at –60 °C.

Radioactivity was measured with an LKB-Wallach scintillation counter (Rackbeta) with automatic quench correction. Ready-Safe (Beckman) was used as scintillator.

RESULTS AND DISCUSSION

Cultures of first-passage human endothelial cells were incubated for 48 h with either ³⁵SO₄²⁻ and [³H]leucine or [³H]leucine alone. Extraction of the cell layer with guanidinium chloride/Triton X-100 removes more than 90% of the ³⁵SO₄²⁻-labelled material, as judged from experiments where the tissue-culture flasks were subsequently extracted with hot (80–100 °C) 0.5 M-NaOH. Proteoglycans from the medium were partially purified by chromatography on DEAE-cellulose before density-

SDS/PAGE (3–8%) of the intact macromolecules and after degradation of polysaccharide side chains

Scheme 1. Flow-chart for the isolation of proteoglycans

Fig. 1. Isopycnic density-gradient centrifugation in 4 M-guanidinium chloride/CsCl of (a) the cell extract and (b) the proteoglycans from the medium of human endothelial cell cultures

Confluent cultures were incubated with $^{35}\text{SO}_4^{2-}$ and $[^3\text{H}]$ leucine for 48 h. The cell layer was extracted with guanidinium chloride, and the proteoglycans from the medium were recovered in guanidinium chloride after partial purification by chromatography on DEAE-cellulose. The samples were adjusted to 4 M-guanidinium chloride and an initial density of 1.31 g/ml by adding water and CsCl. After centrifugation (36000 rev./min for 72 h at 15 °C) the tubes (40 ml) were emptied from the bottom. Fractions (2 ml) were collected and analysed for ^{35}S radioactivity (—) and ^3H radioactivity (·····). The density (----) was measured by weighing portions of alternate fractions.

Fig. 2. Gel-permeation chromatography on Sepharose CL-4B of fractions obtained after isopycnic density-gradient centrifugation

The $^{35}\text{SO}_4^{2-}$ and $[^3\text{H}]$ leucine-labelled materials were obtained as described in the legend to Fig. 1. The column (1 cm $\times$ 100 cm) was eluted with 4 M-guanidinium chloride, and 1 ml fractions were analysed for ^{35}S radioactivity (—), and ^3H radioactivity (·····). In subsequent preparative runs, fractions were pooled as indicated by the bars. (a) Fraction C 1; (b) fraction C 2; (c) fraction M 1; (d) fraction M 2.

gradient centrifugation. The purification procedure is summarized in Scheme 1.

The cell- and medium-derived samples were subjected to isopycnic density-gradient centrifugation in CsCl/4 M-guanidinium chloride (Fig. 1). The proteoglycans from

the cell layer banded mainly at a density of 1.31 g/ml, and little material was found in the bottom fractions. The $^{35}\text{SO}_4^{2-}$ profile of the cell-layer fractions was not different if the density-gradient centrifugation was performed in the absence of Triton X-100. The 'high'

Fig. 3. Ion-exchange h.p.l.c. on Mono Q of proteoglycan fractions obtained by density-gradient centrifugation and gel-permeation chromatography

All fractions were obtained after chromatography on Sepharose CL-4B (Fig. 2), except fraction C 2, which was taken directly from the centrifugation step. The samples were dialysed against the starting buffer. After application, the column was run isocratically for 5 min, after which a linear gradient of 0.3–1.2 M-NaCl (—) (0.5 ml/min) was started. The nominal gradient is indicated in the diagrams. Fractions (0.5 ml) were analysed for ^{35}S radioactivity (—) and ^3H radioactivity (·····). (a) Fraction C 1a after gel chromatography; (b) fraction C 1b after gel chromatography; (c) fraction C 2 after isopycnic density-gradient centrifugation; (d) fraction M 1a after gel chromatography; (e) fraction M 1b after gel chromatography; (f) fraction M 2a after gel chromatography; (g) fraction M 2b after gel chromatography.

density and 'low'-density populations were pooled separately and are referred to below as fractions C 1 and C 2. In the medium, there was more $^{35}\text{SO}_4^{2-}$ -labelled material of 'high' than of 'low' density. These fractions were pooled similarly to those of the cell layer and denoted M 1 and M 2.

Portions of the four fractions were chromatographed on a column of Sepharose CL-4B (Fig. 2). In fraction C 1 three, possibly four, separate $^{35}\text{SO}_4^{2-}$ -labelled macromolecular components were found: C 1a (K_{av} 0.2) and

C 1b (K_{av} 0.5) as well as two more-retarded peaks (K_{av} 0.8 and 0.9). The latter two were not further analysed. Fraction C 2 chromatographed as a single $^{35}\text{SO}_4^{2-}$ -labelled component, which was eluted at K_{av} approx. 0.2. Fraction M 1 was resolved into one minor (M 1a; K_{av} 0.2) and one major (M 1b; K_{av} 0.5) peak of $^{35}\text{SO}_4^{2-}$ -labelled material and, conversely, in fraction M 2 a major population (M 2a) was eluted at K_{av} 0.2 and a minor one (M 2b) at K_{av} 0.5. Fractions containing more than one $^{35}\text{SO}_4^{2-}$ -labelled component (i.e. C 1, M 1

Table 1. Composition and estimated yield of the various proteoglycan fractions

For identification of individual fractions see Figs. 1–3. The contents of $^{35}\text{SO}_4^{2-}$ label in the four fractions obtained after density-gradient centrifugation (i.e. C 1, C 2, M 1 and M 2) were measured and their proportions of the total incorporated $^{35}\text{SO}_4^{2-}$ label were calculated. Three of these fractions (i.e. C 1, M 1 and M 2) resolved into separate peaks of $^{35}\text{SO}_4^{2-}$ -labelled material upon gel chromatography and the relative amounts of ^{35}S radioactivity in these peaks were calculated by integration. The same procedure was applied to the ion-exchange chromatograms, where some fractions (i.e. C 1a, M 1a and M 2b) obtained from gel chromatography separated into two proteoglycan peaks. The relative content of $[^3\text{H}]$ leucine activity in a fraction was calculated by using the relative $^{35}\text{SO}_4^{2-}$ content and the $^{35}\text{SO}_4^{2-}/[^3\text{H}]$ leucine ratio. Major fractions are given in **bold** type. Abbreviations: HS, heparan sulphate; CS, chondroitin sulphate

Fraction	Poly-saccharide chains	$^{35}\text{S}/^3\text{H}$ ratio	^{35}S (% of total)	^3H (% of total)
Cell layer			10	20
C 1			2	
C 1a-Q1	HS	1.5	< 1	< 1
C 1a-Q2	CS	3.9	< 1	< 1
C 1b	CS	4.0	< 1	< 1
C 2	HS	0.46	8	20
Medium			90	80
M 1			40	
M 1a-Q1	HS	2.5	1	< 1
M 1a-Q2	CS	9.3	< 1	< 1
M 1b	CS	2.4	38	18
M 2			38	
M 2a	HS	0.57	30	50
M 2b-Q1	HS and CS	0.88	< 1	1
M 2b-Q2	CS	1.9	8	5

and M 2) were then subjected to preparative gel chromatography on a larger column, and the resulting fractions were pooled in accordance with Fig. 2. Fraction C 2 was not taken through preparative gel filtration.

The seven fractions hereby obtained were further purified by chromatography on a Mono Q ion-exchange column, which was eluted with a linear salt gradient (Fig. 3). During this purification step the remaining non-proteoglycan proteins were removed and the proteoglycans were recovered as one or two peaks of $^{35}\text{SO}_4^{2-}$ -labelled material. In general, these were eluted either at 0.65 M-NaCl or at 0.80 M-NaCl and, as shown below, the early peaks consisted of HSPG and the later ones of CSPG. The two $^{35}\text{SO}_4^{2-}$ -labelled components resolved in fractions C 1a, M 1a and M 2b are referred to below by the fraction code followed by -Q1 and -Q2 for the early and late peaks respectively. Two proteoglycans (C 1a-Q1 and M 1a-Q1) were eluted at an intermediate position (approx. 0.7 M-NaCl) in the gradient. The purified proteoglycan fractions were pooled as indicated in Fig. 3.

The resulting components were analysed for their polysaccharide composition by incubating the proteoglycan with either chondroitin ABC lyase or heparan sulphate lyase followed by chromatography on Sepharose

CL-4B. Degradation of the polysaccharide was indicated by the appearance of the $^{35}\text{SO}_4^{2-}$ -labelled material in the total volume. When degradation with heparan sulphate lyase yielded large fragments, the proteoglycan was redigested with both heparan sulphate lyase and heparin lyase. The results are summarized in Table 1. The glycosaminoglycan chains of the major HSPGs (i.e. fractions C 2 and M 2a) were degraded by heparan sulphate lyase alone, whereas addition of heparin lyase was needed to degrade fractions C 1a-Q1 and M 1a-Q1 to the same extent. This suggests that the latter proteoglycans have longer segments of GlcN(SO₃)-IdoA(SO₄) disaccharide repeats within their polysaccharide chains, as this linkage is not sensitive to cleavage by heparan sulphate lyase. Both heparan sulphate lyase and chondroitin ABC lyase were required to degrade completely the $^{35}\text{SO}_4^{2-}$ -labelled material in M 2b-Q1, showing that this material either is heterogeneous or contains a proteoglycan with both heparan sulphate and chondroitin sulphate chains. The polysaccharides of fractions M 1b and M 2b-Q2 were chondroitin sulphate.

Our estimations of the yield of material in the various fractions are summarized in Table 1. Comparing the contents of radioactivity in the four fractions (C 1, C 2, M 1 and M 2) obtained after density-gradient centrifugation, it is seen that the proteoglycans from the medium comprise 90 % of the total incorporated $^{35}\text{SO}_4^{2-}$ -label. Of the purified proteoglycans, the highest yield of $^{35}\text{SO}_4^{2-}$ radioactivity was obtained in fraction M 1b, followed by fractions M 2a, M 2b-Q2 and C 2. The $^{35}\text{SO}_4^{2-}/[^3\text{H}]$ leucine ratios of the purified proteoglycan fractions differ widely. For example, the ratios are similar for the major HSPG components of the medium and the cell layer (0.57 and 0.46 respectively), but different from that of the CSPGs (e.g. fraction M 1b: 2.4). In addition, the percentage of the total $[^3\text{H}]$ leucine label (among the proteoglycan fractions) in each purified fraction was calculated and the largest proportion was obtained in fraction M 2a, followed by fractions C 2, M 1b and M 2b-Q2. Thus expressing the amounts of proteoglycans as incorporated radiolabel gives different results depending on the precursors used.

To identify the core proteins of the major HSPG fractions (C 2 and M 2a), they were subjected to SDS/PAGE, both as intact proteoglycans and after treatment with heparan sulphate lyase (Fig. 4). To allow safe assessment of purity and identity of the core proteins, we used proteoglycans labelled with only $[^3\text{H}]$ leucine in all electrophoreses. They were purified by the same procedures as used for the $^{35}\text{SO}_4^{2-}$ - and $[^3\text{H}]$ leucine-labelled fractions shown above. The intact proteoglycans barely entered the gels, indicating an apparent molecular mass of at least 600–800 kDa (Fig. 4, lanes a, c, e and g). In both fractions C 2 and M 2a a broad band with apparent molecular mass approx. 300 kDa appeared after digestion with heparan sulphate lyase (Fig. 4, lanes b and f). In fraction C 2 this was the major core protein, whereas in fraction M 2a there emerged an additional protein core with apparent molecular mass approx. 210 kDa (Fig. 4, lane f). Both fraction C 2 and fraction M 2a contained a less prominent core protein with apparent molecular mass approx. 280 kDa (Fig. 4, lanes b and f). Separate runs showed that the major core protein migrated further than non-reduced plasma fibronectin (440 kDa; results not shown). When the samples were reduced with 2-mercaptoethanol, the apparent molecular mass of the

Fig. 4. SDS/PAGE of the major heparan sulphate proteoglycans as intact molecules and after treatment with heparan sulphate lyase

Endothelial cells were metabolically labelled with [^3H]leucine (20 $\mu\text{Ci}/\text{ml}$) for 48 h. Fractions C 2 and M 2a from ion-exchange h.p.l.c. (Fig. 3) were concentrated on DEAE-cellulose and dialysed against 3 mM-calcium acetate/0.1 % Triton X-100/10 mM-Hepes buffer, pH 7.0. Equal amounts of radioactivity were applied to each lane. Electrophoresis was run on 3–8 % gradient polyacrylamide gels at a constant current of 12 mA/gel overnight. The fluorographs shown are: lane a, fraction C 2 non-reduced; lane b, fraction C 2 non-reduced and digested with heparan sulphate lyase; lane c, fraction C 2 reduced with 2-mercaptoethanol; lane d, fraction C 2 digested with heparan sulphate lyase and reduced; lane e, fraction M 2a non-reduced; lane f, fraction M 2a non-reduced and digested with heparan sulphate lyase; lane g, fraction M 2a reduced; lane h, fraction M 2a digested with heparan sulphate lyase and reduced. The migration distances of standard proteins are indicated. The molecular-mass values were estimated from a plot of $\log(\text{molecular mass})$ versus migration distance.

main core protein increased from approx. 300 kDa to 350 kDa (Fig. 4, lanes d and h). The heterogeneity of the core protein was also more pronounced after reduction, with the appearance of one or two additional minor bands. The presence of the latter may reflect incidental proteolytic cleavage within a loop formed by an intra-chain disulphide bond near one end of the polypeptide. When the core proteins in fraction M 2a were reduced (Fig. 4, lane h), the 210 kDa component increased to approx. 250 kDa. In this fraction (Fig. 4, lanes e–h), a faint band appearing at molecular mass approx. 180 kDa was not sensitive to digestion with heparan sulphate lyase, thus representing a contaminating protein. In a preliminary experiment, the proteoglycan in fraction M 1a-Q1 was subjected to SDS/PAGE before and after digestion with heparan sulphate lyase. The pattern of core proteins was similar to that of fraction M 2a (cf. Fig. 4, lane h), with a triplet at about 300 kDa and a single band at 250 kDa (reduced samples, results not shown).

To test further whether the intact HSPGs are sensitive to reduction, samples of fractions C 2 and M 2a were chromatographed on Sepharose CL-4B after reduction of disulphide bonds. The reduced components displayed a slight broadening of the peak towards a more included volume (results not shown). The experiments confirm that these HSPG fractions contain monomeric protein cores.

Fig. 5. SDS/PAGE of the purified chondroitin sulphate proteoglycans as intact molecules and after treatment with chondroitin ABC lyase

Endothelial cells were metabolically labelled with [^3H]leucine for 48 h. Fractions M 1b and M 2b obtained by ion-exchange chromatography were concentrated on DEAE-cellulose and dialysed against 0.1 % Triton X-100/10 mM- Na_2EDTA /10 mM-Tris/HCl buffer, pH 7.3. Electrophoresis was run on 3–12 % gradient polyacrylamide gels at a constant current of 12 mA overnight. The fluorographs shown are: lane a, fraction M 1b; lane b, fraction M 1b after digestion with chondroitin sulphate ABC lyase; lane c, fraction M 2b; lane d, fraction M 2b after digestion with chondroitin ABC lyase. All samples were reduced with 2-mercaptoethanol.

The proteoglycans of fractions C 2 and M 2a were tested for lipophilic properties by hydrophobic chromatography on octyl-Sepharose CL-4B after removal of excess detergent. Under these conditions, more than 95 % of the material bound to octyl-Sepharose (results not shown).

The size and homogeneity of the polysaccharide chains was studied by gel chromatography after subjecting purified proteoglycan fractions to alkaline elimination. Fractions corresponding to C 2 and M 2a afforded polysaccharide chains that eluted as a unimodal peak with K_{av} 0.55 on Sepharose CL-4B (results not shown).

The sizes of the different purified CSPGs were also determined by SDS/PAGE, both of the intact proteoglycans after digestion with chondroitin ABC lyase (Fig. 5). The major CSPG (M 1b) appeared homogeneous with apparent molecular mass approx. 200 kDa (Fig. 5, lane a) and a core protein of 47 kDa. The less prevalent fraction M 2b-Q2 had a lower molecular-mass value, approx. 80–100 kDa (Fig. 5, lane c), but a similar core protein (Fig. 5, lane d). In fraction M 2B-Q2 traces of a larger proteoglycan were seen as a trailing tail in the fluorogram.

GENERAL DISCUSSION

The major proteoglycan found in the cell layer, including both membrane-associated and matrix-associated molecules, is a large HSPG, in agreement with results obtained by previous investigators [2–4,8]. The HSPG has a molecular mass of at least 600 kDa (SDS/PAGE) and a buoyant density of 1.31 g/ml, the latter finding suggesting a relatively high protein content. The core protein appears to be a monomer with a molecular mass of approx. 350 kDa. The presence of minor components with lower molecular-mass values might be due to proteolytic modification of the large core, either *in situ* or during preparation. However, the core-protein pattern does not change during 24–72 h of metabolic labelling, and the same results were obtained with various purification procedures used during the development of the present study (results not shown). Other causes of the heterogeneity, such as different extents of *N*- and *O*-glycosylation, are possible. The size of the major core protein seemingly increased upon reduction, suggesting the presence of intrachain disulphide bonds. The endothelial HSPG thus resembles that isolated from basement-membrane-producing EHS tumours (for a review see ref. [20]), both regarding buoyant density and the large size of the core protein.

The large endothelial HSPG has affinity for octyl-Sepharose, which might suggest that it is anchored in the plasma membrane. However, treatment of the cell layer with a detergent-containing physiological buffer, such as phosphate-buffered saline, causes release of only a small amount of this proteoglycan, and, unless certain precautions are taken, this extraction procedure results in partial degradation of the heparan sulphate side chains (A. Lindblom & L.-Å. Fransson, unpublished work).

Assuming that the large HSPG of the medium (M 2a) is related to that of the cell layer (C 2), and comparing the amounts of the proteoglycan in the respective compartments (Table 1), it seems that the HSPG is partially retained at the cell layer. In contrast, nearly all of the CSPGs are found in the medium (Table 1). The exact cellular localization, for example the plasma membrane

or the pericellular matrix, of the endothelial HSPG remains to be determined. In human lung fibroblasts a similar proteoglycan is associated with fibres in the subcellular matrix and not with the plasma membrane [21]. The core protein in the medium HSPG (M 2a) is more heterogeneous than that of the cell-layer HSPG (C 2), and comprises an additional component of molecular mass 250 kDa which is absent in the cell-layer HSPG. This smaller core may occur as a result of proteolytic cleavage *in situ*, as described for HSPGs from glomeruli [22].

Marcum *et al.* [8] found that less than 1 % of the large HSPG from monolayers of cloned bovine aortic endothelial cells had antithrombin-cofactor activity. This small proportion could correspond to the amount of HSPG found here, both in the cell layer and the medium within the high-buoyant-density fractions (fractions C 1a-Q1 and M 1a-Q1; Table 1). But, in contrast with the major part of HSPG of the cloned cells, the antithrombin-binding proteoglycan had affinity for octyl-Sepharose. This finding apparently conflicts with our results, which show that all of the major human endothelial HSPGs bind to octyl-Sepharose. However, the conditions that we used for chromatography (see the Experimental section) were different from the protocol for Marcum *et al.* [8].

A significant part of the $^{35}\text{SO}_4^{2-}$ -labelled material from the medium consists of a high-density CSPG (M 1b) with a high $^{35}\text{SO}_4^{2-}/[^3\text{H}]\text{leucine}$ ratio (Table 1), a higher negative charge density and a smaller overall size than the large HSPG (Fig. 3). This proteoglycan has a molecular mass of 180–200 kDa and a core protein with a molecular mass of 47 kDa. Furthermore, a small amount of a CSPG (M 2b-Q2) with a lower molecular-mass value (80–100 kDa) and containing a similar core protein was found in the medium. These two CSPGs bear resemblance to proteoglycans PG S1 and PG S2 previously isolated from cartilage and other tissues [23]. Proteoglycan PG S1, which is thought to carry more or longer side chains, is present in nasal cartilage and aortic intima. Proteoglycan PG S2 is found in articular cartilage and in many fibrous connective tissues, where it interacts with collagen fibres [24]. Both proteoglycans are major components in fibroblast and smooth-muscle cell cultures [25–27]. The core proteins of proteoglycans PG S1 and PG S2 have recently been shown to be homologous but distinct, in spite of having the same or similar molecular mass on SDS/PAGE [28].

Some proteoglycan fractions (C 1a-Q1, C 1a-Q2 and C 1b from the cell layer and M 1a-Q2 and M 2b-Q1 from the medium) were not obtained in sufficient quantities to permit core-protein analyses. Possibly, a galactosaminoglycan-substituted thrombomodulin component [29] could be present in these fractions. In contrast with other investigators [2–4], we did not detect any 'small' heparan sulphate proteoglycans in either the cell extracts or the spent media, possibly owing to differences in methodology or between human and bovine cells. Furthermore, we did not find any distinct proteoglycan species associated with the plasma membrane as described for human skin [30] and lung fibroblasts [31]. Human embryonic fibroblasts also synthesize a large CSPG containing a core protein of molecular mass 450 kDa on SDS/PAGE [25], which is not present in early-passage endothelial cultures.

In summary, endothelial cells synthesize two major

classes of proteoglycan core proteins. One is large (apparent molecular mass 350 kDa) and carries heparan sulphate side chains that, except for a small subfraction (fractions C 1a-Q1 and M 1a-Q1), are extensively degraded by heparan sulphate lyase. The other core protein is small (apparent molecular mass 47 kDa) and carries chondroitin sulphate side chains of varied amount and/or length that give rise to two subclasses: a predominant 200 kDa proteoglycan and a less prevalent 100 kDa form. The endothelial HSPG is present in both the culture monolayer and the medium, whereas the CSPG is found almost exclusively in the medium.

This work was supported by grants from the Swedish Medical Research Council (567), the National Swedish Board for Technical Development (1543), the Swedish Cancer Society (2545), Gustav V 80-Årsfond, Lundbergs Stiftelse, John och Augusta Perssons Stiftelse, Svenska Sällskapet för Medicinsk Forskning, Österlunds Stiftelse and the Medical Faculty, University of Lund. We are grateful to J. Hernäs, L. Cöster, A. Franzén, T. Larsson, A. Malmström, Å. Oldberg and Y. Sommarin for valuable discussions and helpful advice, to Mrs. B. Havsmark for technical assistance and to Ms. B. Jönsson for art work. For supply of umbilical cords we are indebted to the Delivery Care Unit, Lund University Hospital.

REFERENCES

1. Marcum, J. A. & Rosenberg, R. D. (1984) *Biochemistry* **23**, 1730–1737
2. Kramer, R. H., Vogel, K. G. & Nicholson, G. L. (1982) *J. Biol. Chem.* **257**, 2678–2686
3. Oohira, A., Wight, T. N. & Bornstein, P. (1983) *J. Biol. Chem.* **258**, 2014–2021
4. Kinsella, M. G. & Wight, T. N. (1988) *Biochemistry* **27**, 2136–2144
5. Marcum, J. A. & Rosenberg, R. D. (1987) *Semin. Thromb. Hemostasis* **13**, 464–474
6. Jaffe, E. A. (ed.) (1984) *Biology of Endothelial Cells*, Martinus Nijhoff, Boston
7. Shimada, K. & Ozawa, T. (1985) *J. Clin. Invest.* **75**, 1308–1316
8. Marcum, J., Atha, D. H., Fritze, L. M. S., Nawroth, P., Stern, D. & Rosenberg, R. D. (1986) *J. Biol. Chem.* **261**, 7507–7517
9. Esmon, C. T. & Esmon, N. L. (1984) *Semin. Thromb. Hemostasis* **10**, 122–130
10. Suzuki, K., Kusumoto, H., Deyashiki, Y., Nishioka, J., Maruyama, I., Zushi, M., Kawahara, S., Honda, G., Yamamoto, S. & Horiguchi, S. (1987) *EMBO J.* **6**, 1891–1897
11. Jackman, R. W., Beeler, D. L., Fritze, L., Soff, G. & Rosenberg, R. D. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6425–6429
12. Olivecrona, T., Bengtsson, G., Marklund, S. E., Lindahl, U. & Höök, M. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 60–65
13. Shimada, K., Gill, P. G., Silbert, J. E., Douglas, W. H. J. & Fanburg, B. L. (1981) *J. Clin. Invest.* **68**, 995–1002
14. Sakela, O., Moscatelli, D., Sommer, A. & Rifkin, D. B. (1988) *J. Cell Biol.* **107**, 743–751
15. Engvall, E. & Ruoslahti, E. (1977) *Int. J. Cancer* **20**, 1–5
16. Jaffe, E. A., Nachman, R. L., Becker, C. G. & Minick, C. R. (1973) *J. Clin. Invest.* **52**, 2745–2756
17. Jaffe, E. A., Hoyer, L. W. & Nachman, R. L. (1973) *J. Clin. Invest.* **52**, 2757–2764
18. Shively, J. E. & Conrad, H. E. (1976) *Biochemistry* **15**, 3932–3942
19. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
20. Hassel, J. R., Kimura, J. H. & Hascall, V. C. (1986) *Annu. Rev. Biochem.* **55**, 539–567
21. Heremans, A., Cassiman, J.-J., Van Den Berghe, H. & David, G. (1988) *J. Biol. Chem.* **263**, 4731–4739
22. Klein, D. J., Brown, D. M., Oegema, T. R., Branchley, P. E., Anderson, J. C., Dickinson, M. A. J., Horigan, E. A. & Hassel, J. R. (1988) *J. Cell Biol.* **106**, 963–970
23. Heinegård, D. & Sommarin, Y. (1987) *Methods Enzymol.* **144**, 305–319
24. Scott, J. E. (1988) *Biochem. J.* **252**, 313–323
25. Cöster, L., Carlstedt, I., Malmström, A. & Särnstrand, B. (1984) *Biochem. J.* **220**, 575–582
26. Glössl, J., Beck, M. & Kresse, H. (1984) *J. Biol. Chem.* **259**, 14144–14150
27. Rauch, U., Glössl, J. & Kresse, H. (1986) *Biochem. J.* **238**, 465–474
28. Fisher, L. W., Termine, J. D. & Young, M. F. (1989) *J. Biol. Chem.* **264**, 4571–4576
29. Bourin, M.-C., Boffa, M.-C., Björk, I. & Lindahl, U. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5924–5928
30. Cöster, L., Carlstedt, I., Kendall, S., Malmström, A., Schmidtchen, A. & Fransson, L.-Å. (1986) *J. Biol. Chem.* **261**, 12079–12088
31. Lories, V., David, G., Cassiman, J.-J. & Van den Berghe, H. (1986) *Eur. J. Biochem.* **158**, 351–359

Received 16 November 1988/23 January 1989; accepted 31 January 1989