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Domain structure of endothelial heparan sulphate

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The domain structure of heparan sulphate chains from an endothelial low-density proteoglycan was examined using specific degradations of the chains while attached to the intact proteoglycan. 'Inner' chain fragments, remaining on the protein core, were separated from 'outer' fragments by gel chromatography, and were subsequently released from the protein core by alkaline cleavage. The structure of 'inner' and 'outer' chain fragments was then examined and compared. Using deaminative cleavage we obtained evidence that the first *N*-sulphated glucosamine residue is variably positioned some 10–17 disaccharides from the xylose–serine linkage of the proteoglycan. Digestion with heparinase yielded 'inner' and 'outer' fragments covering a broad range of different sizes, indicating a scarce and variable distribution of sulphated iduronic acid in the native chains. *N*-sulphated glucosamine occurred more frequently in the 'outer' fragments. We also studied the affinity of the endothelial heparan sulphate chains towards two presumptive biological ligands, namely antithrombin III and lipoprotein lipase. A major part of the endothelial heparan sulphate chains showed a weak affinity for antithrombin III and the affinity was essentially lost on heparinase digestion. On lipoprotein lipase–agarose the endothelial heparan sulphate chains were eluted at the same salt concentration as heparin, and the binding persisted, although with decreased strength, after digestion with heparinase.

INTRODUCTION

Heparan sulphate proteoglycans (HSPGs) are a heterogeneous family of complex macromolecules containing a core protein with covalently linked polyanionic glycosaminoglycan chains. Several core proteins, constituting different gene products, have been described [for a review see Gallagher (1989)]. Some HSPGs are integral components of the plasma membrane (Lories *et al.*, 1989) whereas others are located in the extracellular matrix or basement membranes (Heremans *et al.*, 1989). The diversity of these proteoglycans implies different functions, and they have also been found to take part in several biological processes. For example, their fixed negative charges might improve the molecular filter function of the kidney glomeruli (Kanwar *et al.*, 1980) and of other basement membranes. Other proposed functions of heparan sulphate (HS) include inhibition of blood coagulation (de Agostini *et al.*, 1990), binding of growth factors (Saksela & Rifkin, 1990), anchoring of lipoprotein lipase (Olivecrona & Bengtsson-Olivecrona, 1989) and interaction with extracellular matrix proteins [laminin, fibronectin, collagen and thrombospondin; for references see Lane (1989)]. The most studied interaction of heparin or HS is that with antithrombin III. High-affinity binding, as well as anticoagulant effect, requires the presence of a specific pentasaccharide sequence (see, e.g. Thunberg *et al.*, 1982). In the interaction of heparin or HS with other proteins the structural requirements seem less stringent (Bengtsson *et al.*, 1980; Lane, 1989).

Detailed knowledge of the structure and biosynthesis of the glycosaminoglycans has mostly been based on studies of heparin and microsomal preparations from mastocytomas [for references see Lindahl (1989)]. Both heparin and HS are first synthesized as repetitive polymers of alternating GlcNAc- and GlcA-residues. Subsequently the chains undergo a series of modifications starting with *N*-deacetylation and *N*-sulphation of the glucosamines (Lindahl, 1989). The major difference between HS and heparin lies in the degree of *N*-sulphation, where the former usually has more than 50% of the glucosamines *N*-acetylated and heparin less than 20% (Gallagher & Lyon, 1989). Long *N*-acetylated segments are characteristic for HS and these chains can be regarded as sequences of alternating *N*-acetylated and *N*-sulphated 'domains', constituting regions of lower and higher charge density.

In previous work, we have characterized the major proteoglycans synthesized by human endothelial cells (Lindblom *et al.*, 1989). We have also analysed the disaccharide composition of the chains derived from the major HSPG (M_r 600000–800000) synthesized by these cells (Lindblom & Fransson, 1990). In the present study the architecture of these chains is examined in more detail. The intact HSPG {fraction M 2a derived from the spent medium of endothelial cell cultures [the codes used for the proteoglycans are from Lindblom *et al.* (1989) and denote high-density (> 1.4 g/ml) dermatan sulphate proteoglycan (DSPG) from the spent medium (M 1b) and low-density (1.31 g/ml) HSPG from the spent medium (M 2a)]} was degraded by using either deaminative cleavage at $GlcNSO_3$- residues (Shively & Conrad, 1976) or heparinase cleavage of bonds to iduronic acid 2-sulphate) [IdoA(2S)] (Lindhardt *et al.*, 1990), followed by separation of the core protein-attached 'inner' fragments from 'outer' ones by gel chromatography. By this means we could identify a polydisperse but homogeneous region of contiguous *N*-acetylated disaccharides after the linkage-region tetrasaccharide GlcA-Gal-Gal-Xyl (Fransson, 1985). Both 'inner' and 'outer' fragments isolated after heparinase digestion were heterogeneous, and the smallest 'inner' fragments were approximately the same size as the *N*-acetylated 'inner' fragments after deaminative cleavage. The affinity of antithrombin III and

Abbreviations used: HSPG, heparan sulphate proteoglycan; HS, heparan sulphate; DSPG, dermatan sulphate proteoglycan; DS, dermatan sulphate; d.p., degree of polymerization (a disaccharide has d.p. of 1, etc.); aMan, anhydromannose; GlcNAc, *N*-acetylated glucosamine; $GlcNSO_3$, *N*-sulphated glucosamine; GlcNR6R, monosulphated glucosamine; GlcA, D-glucuronic acid; IdoA, L-iduronic acid; IdoA(2S), iduronic acid 2-sulphate; HexA, hexuronic acid; ΔHexA, hexuronic acid with a 4,5-double bond; ΔHexA(2S), 2-sulphated hexuronic acid with a 4,5-double bond; Xyl, xylose.

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lipoprotein lipase of the intact endothelial heparan sulphate chains versus isolated 'inner' and 'outer' heparinase fragments was also assessed using affinity chromatography.

EXPERIMENTAL

Materials

Hyaluronic acid (Healon), Q-Sepharose Fast Flow, a Fast Desalting (HR 10/10) column and a Superose 6 (HR 10/30) column were obtained from Pharmacia-LKB, Uppsala, Sweden; neuraminidase (from *Clostridium perfringens*, Type X) was from Sigma; TSK-Fractogel HW40 and pyridine GR were from Merck; dialysis membranes (Spectrapor 1 and Spectrapor 3; cut-off 6000–8000 and 3500 respectively) were from Spectrum, Los Angeles, CA, U.S.A.; a microdialysis system was from Bethesda Research Laboratories, Gaithersburg, MD, U.S.A. Anti-thrombin III–Sepharose, prepared as described by Höök *et al.* (1976), was provided by Dr. L.-O. Andersson, AB Kabi Vitrum, Stockholm, Sweden. Conductivity was measured with a model 101 Orion research conductivity meter. Endothelial cells were prepared from human umbilical veins and cultured on fibronectin-coated dishes as described (Lindblom *et al.*, 1989). Cells within the first two passages (split ratio 1:3) were used for metabolic labelling with [^{3}H]glucosamine (20 μCi/ml). Labelled proteoglycans from the cell extract and spent medium were isolated by isopycnic density gradient centrifugation, gel filtration and ion-exchange f.p.l.c. as described (Lindblom *et al.*, 1989). Oligosaccharide standards were non-sulphated (ΔHexA1-4GlcNAc) and monosulphated disaccharide (ΔHexA1-4GlcNR6R) obtained by heparitinase digestion of a low-sulphated heparan sulphate (bovine lung), and tri-sulphated disaccharide [ΔHexA(2S)1-4GlcNSO$_3$(6S)] obtained by heparinase-digestion of heparin. The standards were purified by gel chromatography on Bio-Gel P2 (Lindblom & Fransson, 1990). Other materials were from sources listed previously (Lindblom *et al.*, 1989; Lindblom & Fransson, 1990).

Degradative methods

For deaminative cleavage of chains in the intact HSPG, a sample (approx. 2×10^6 ^{3}H d.p.m.), containing 1 mg of dextran (M_r 500000) as carrier, was dialysed against water (1000 vol., two changes) and freeze-dried in a Speed-Vac centrifuge (Heto, Denmark). The HSPG was then treated with 50 μl of 0.5 M-HNO_2 for 10 min on a shaker at room temperature, and the reaction terminated by the addition of 7.5 μl of 2 M-Na_2CO_3 (Shively & Conrad, 1976). The core protein, with the proximal *N*-acetylated fragments of heparan sulphate chains still attached, was separated from oligosaccharide fragments by gel. f.p.l.c. on Superose 6 (conducted as described in the legend to Fig. 1) and subjected to alkaline elimination (see below). The sample was then neutralized and partially desalted on a Fast Desalting column connected to a Pharmacia-LKB f.p.l.c. system. The column (15 ml) was run at 1 ml/min in 0.5 M-NH_4HCO_3. The radioactive material was pooled, freeze-dried, redissolved in water and subjected to ion-exchange chromatography on Q-Sepharose Fast Flow (see below).

Another sample of purified HSPG (containing 1 mg of dextran as carrier) was dialysed against 3 mM-calcium acetate/0.1 % (v/v) Triton X-100/10 mM-Hepes/NaOH, pH 7.0. The dialysed sample was supplemented with 0.1 vol. of 1 % (w/v) ovomucoid in the same buffer and digested with heparinase (4 munits/ml) for 24 h at 30 °C. Samples were taken at different time intervals (0, 6, 9 and 24 h) to monitor the degradation by gel f.p.l.c. on Superose 6.

When the amount of oligosaccharides had reached a plateau (complete degradation, cf. Fig. 4), the digested HSPG sample was desiccated in a Speed-Vac centrifuge, redissolved in 200 μl of 4 M-guanidinium chloride/0.1 % (v/v) mulgophene/50 mM-sodium acetate buffer, pH 5.8, and applied to the Superose 6 column, where oligosaccharides ('outer' fragments) separated from the core protein. Pooled fractions were then dialysed (Spectrapor 3) against 6 M-urea/0.1 % (v/v) Triton X-100/10 mM-Tris/HCl, pH 8.0, and applied to a column (0.5 ml) of DEAE-cellulose DE-53, equilibrated in the same buffer. After being washed with equilibrating buffer (5 ml), the radioactive material (> 90 %) was eluted with 4 M-guanidinium chloride/0.2 % (v/v) Triton X-100/50 mM-sodium acetate, pH 5.8.

HS chains, either intact or degraded, were liberated from the core protein by alkaline elimination. Freeze-dried samples were dissolved in 0.1 M-$NaBH_4$/0.5 M-NaOH and incubated overnight at room temperature. The samples were then neutralized by the addition of 0.5 M-acetic acid, and either partially desalted on a Fast Desalting column (see above), or diluted (1:10) in 6 M-urea/0.1 % (v/v) Triton X-100/10 mM-Tris/HCl, pH 8.0, and applied to a column (0.5 ml) of DEAE-cellulose DE-53, equilibrated in the same buffer. The column was washed with equilibrating buffer (5 ml), followed by 5 ml of 6 M-urea/0.1 % (v/v) Triton X-100/0.2 M-sodium acetate, pH 5.8, and then 5 ml of 6 M-urea/0.1 % (v/v) Triton X-100/0.5 M-sodium acetate, pH 5.8 (this step was always omitted in the purification of degraded chains). Finally, the glycosaminoglycan material was eluted with 4 M-guanidinium chloride/0.2 % (v/v) Triton X-100/50 mM-sodium acetate, pH 5.8.

Digestion with heparitinase was performed with 15 munits of enzyme in 100 μl of 3 mM-calcium acetate/0.1 M-sodium acetate, pH 7.0, containing 50 μg of heparan sulphate carrier (from bovine lung). For digestion with neuraminidase (0.75 μg of enzyme), the sample was redissolved in 10 μl of 0.05 M-sodium acetate, pH 5, containing 50 μg of BSA, and incubated at 37 °C for 24 h.

Ion-exchange chromatography

A column (5 ml) of Q-Sepharose Fast Flow was equilibrated in 2 mM-pyridine acetate, pH 5.3. Before application, samples were diluted to the same conductivity as the equilibration buffer (approx. 70 μS). The column was eluted with linear gradients of 2 mM–2 M- and 2–4 M-pyridine acetate, mixed by a Pharmacia-LKB f.p.l.c. system (Lindblom *et al.*, 1989). After each run, the column was washed with 5 ml of 2 M-NH_4HCO_3, followed by 5 ml of 2 M-NaOH, after which the column was re-equilibrated in 2 mM-pyridine acetate. The flow was 0.5 ml/min and 1 ml fractions were collected. Strong anion-exchange (SAX) h.p.l.c. was performed on a Varian model 5560 system fitted with a Partisil 10/25 SAX column (Whatman) as described (Lindblom & Fransson, 1990). Oligosaccharide samples (200–1000 μl) diluted to less than 1 mM-salt were injected and the column eluted with water for 10 min, followed by a linear gradient from 0 to 0.6 M-KH_2PO_4, pH 4.2, in 60 min. Flow was 1 ml/min and 1 ml fractions were collected. In each run, disaccharide standards (see the Experimental section) were used and their elution was monitored by measuring A_{232}. A Pharmacia HR 5/5 Mono Q column was eluted with a linear gradient of 0–1.2 M-NaCl in 7 M-urea/0.1 % (v/v) Triton X-100/10 mM-Tris/HCl, pH 8.0, as described previously (Lindblom *et al.*, 1989).

PAGE and electroblotting

Electrophoresis of saccharides was performed on linear gradient gels (20–30 % (w/v) acrylamide/0.5–3.6 % cross-linker) as described by Fransson *et al.* (1990). Semi-dry electroblotting of electrophoretically separated oligosaccharides was achieved as described (Lindblom & Fransson, 1990). The dried membranes

were sprayed with Amplify (Amersham International), briefly dried and exposed to a film at −60 °C for 7–20 days.

Antithrombin III chromatography

The capacity of the antithrombin gel to bind heparin was checked by applying 5 mg of heparin in 1 ml of 50 mM-Tris/HCl, pH 7.5, and then washing the column (2 ml gel) stepwise with (a) 8 ml of sample buffer, (b) 8 ml of 0.20 M-NaCl/50 mM-Tris/HCl, pH 7.5, and finally (c) 8 ml of 3 M-NaCl/50 mM-Tris/HCl, pH 7.5. Fractions of 1 ml were collected and analysed for heparin content by a Dimethylene Blue assay described by Farndale *et al.* (1982) and using heparin as standard. Heparin eluted in the 3 M-NaCl step (essentially constituting high-affinity heparin) was always eluted with the same concentration when reapplied (after dialysis) to the column, indicating that binding was specific. Of the 5 mg applied to the affinity column, approximately 2 mg was eluted in 3 M-NaCl.

Radioactive samples, containing 50 μg of heparan sulphate (bovine lung) as carrier, were dissolved in 500 μl of 50 mM-Tris/HCl buffer, pH 7.5, and applied to the column. After sample application, the column was eluted using a slow flow (2 ml/h) for 30 min, after which the flow was increased (6 ml/h) for the rest of the run. At least 6 ml was used in each elution step and fractions of 1 ml were collected.

Lipoprotein lipase–agarose chromatography

Lipoprotein lipase–agarose was made by coupling lipoprotein lipase to CNBR-activated Sepharose 4B, as detailed elsewhere (Liu *et al.*, 1991). The lipase–agarose was used only once and within 2 weeks after preparation. When comparing the affinity of different glycan preparations for the immobilized lipase, experiments were performed in parallel on the same day. Columns of 0.5 ml (inner diameter 5 mm) were washed with 2 M-NaCl/20 mM-Tris/HCl, pH 7.4, containing 0.1 % (v/v) Triton X-100, and then equilibrated in 10 mM-Bistris, pH 6.5. Typically 10 μl of the glycans, in 4 M-guanidinium chloride/0.2 % (v/v) Triton X-100/50 mM-sodium acetate, pH 5.8, were diluted in 2 ml of the equilibration buffer supplemented with 1 mg of BSA/ml. The sample was applied (0.1 ml/min) and the column was then washed with 10 ml of equilibration buffer (0.5 ml/min). Elution was performed with a linear gradient of 0.1–1 M-NaCl (10 ml + 10 ml, 0.5 ml/min). Fractions were collected directly into vials for liquid-scintillation counting (two fractions/min). A sample (25 μl) from each fraction was diluted in water for determination of the concentration of NaCl by conductometry (comparison of standard solutions made up in the same buffer). The recovery of radioactivity over the columns were usually better than 95 % and binding was usually better than 90 %.

Fractions eluted from gel chromatography, ion-exchange and affinity chromatography were analysed for ^{3}H radioactivity in a scintillation counter from LKB–Wallach using Ready-Safe (Beckman) as a scintillator.

RESULTS AND DISCUSSION

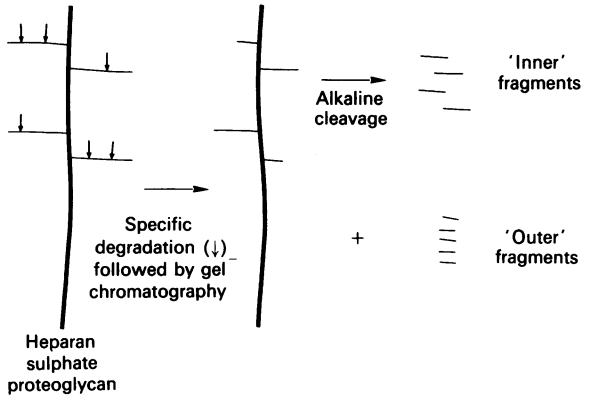
Deaminative cleavage of the glycan chains

Specific degradation of glycosaminoglycans linked to an intact proteoglycan should leave the reducing end fragments of the original chains still attached to the core protein. As illustrated in Scheme 1, the 'outer' fragments can then be separated from the large core protein containing the 'inner' fragments. Intact [^{3}H]glucosamine-labelled low-density HSPG was treated with HNO_2, which cleaves HS chains at the $GlcNSO_3$- residues. When the degraded sample was chromatographed on Superose 6 (Fig. 1), approximately 15 % of the [^{3}H]glucosamine label was eluted near the void volume and the remaining label was eluted close to the total volume, representing oligosaccharide fragments of the general structure HexA-[GlcNAc-HexA]$_n$-aMan. The fractions eluted near the void volume, containing core protein with the proximal *N*-acetylated fragments of HS chains still attached, were pooled (see Fig. 1) and the chain fragments were released by alkaline elimination. After neutralization and desalting (see the Experimental section), the sample was applied to a column of Q-Sepharose Fast Flow, eluted with linear gradients of pyridine acetate, pH 5.3 (see Fig. 2). At this pH, most peptides will be positively charged or neutral, whereas oligosaccharides containing sialic acid, uronic acid, or SO_4^{2-}, will be negatively charged. Even unsulphated heparan sulphate tetrasaccharides quantitatively bind to the column in 2 mM-pyridine acetate (A. Lindblom, unpublished work). Almost all the [^{3}H]glucosamine-labelled material was bound to the column. Approximately 50 % was eluted at the start of the gradient (QS1, Fig. 2), whereas the rest was eluted as a rather homogeneous peak at about 0.8 M-pyridine acetate (QS2, Fig. 2). The two peaks were pooled, freeze-dried and analysed by gel chromatography.

As judged from gel chromatography on Sepharose CL 6B and Bio-Gel P6, the early eluted material from Q-Sepharose (QS1) contained heterogeneous low-M_r material. Results from h.p.l.c. SAX before and after treatment with neuraminidase suggest that the negative charge of the material in QS1 is contributed largely, if not solely, by sialic acid (results not shown).

The material in the late peak from Q-Sepharose (QS2) was eluted as a rather homogeneous population on CL 6B (Fig. 3*a*). According to the calibration of Sepharose 6B with chondroitin sulphate standards by Wastesson (1971), the elution position of QS2 indicates an average M_r of about 5000, corresponding to oligosaccharides of d.p. 12–13 (M_r of GlcAβ1–4GlcNAc in a polymer is 395 and M_r of the linkage tetrasaccharide is 695). The early shoulder on the profile in Fig. 3(*a*) suggests the presence of larger fragments. On Bio-Gel P6, QS2 was eluted in the void volume (Fig. 3*b*), which further indicates that the size of the fragment is not smaller than d.p. 8. The homogeneous low charge density and the molecular size of QS2 suggests that it was an unsulphated HS fragment. Its general structure should be HexA-GlcNAc-[GlcA-GlcNAc]-GlcA-Gal-Gal-Xyl, since it must contain the linkage-region tetrasaccharide (see Fransson, 1985) and since *O*-sulphation and C-5 epimerization of glucuronic acid (into iduronic acid) requires the presence of an adjacent $GlcNSO_3$- residue (see Lindahl, 1989). From what is known of the specificity of heparitinase, it cleaves the hexosaminidic bonds in disaccharides of the type GlcNR6Rα1-4GlcA (Lindhardt *et al.*, 1990). Thus, if our structural predictions were correct, this enzyme should degrade QS2 into disaccharides (apart from the linkage-region tetrasaccharide). However, as seen in Fig. 3(*c*), heparitinase digestion of QS2 yielded oligosaccharides ranging from d.p. 1 to approximately 8, in spite of the use of a large amount of enzyme (smaller amounts gave very little or no detectable degradation; A. Lindblom, unpublished work). This is probably explained by the earlier finding that shorter unsulphated heparan chains are poor substrates for heparitinase (Silverberg *et al.*, 1985).

In a separate experiment, alkali-released material from the HNO_2-treated proteoglycan was recovered by stepwise elution from DEAE-cellulose (see the Experiment section). The material recovered in this procedure should be essentially equivalent to QS2. This sample was subjected to gradient PAGE (Fig. 3*d*, lane 1). For a reference, DS chains (derived from an endothelial high-density DSPG, M 1b) digested with chondroitinase AC I was run in a parallel lane (Fig. 3*d*, lane 2). Chondroitinase AC I cleaves hexosaminidic bonds to glucuronic acid in chondroitin or DS, yielding oligosaccharides of d.p. 1 to approximately 30 when cleaving endothelial DS. The oligosaccharide material derived



Scheme. 1. Degradation of HS chains and separation of 'inner' (reducing-end) and 'outer' (non-reducing) fragments

After selective cleavage, the reducing-end fragments remain attached to the large core protein and can be separated from smaller non-reducing-end fragments, e.g. by gel chromatography.

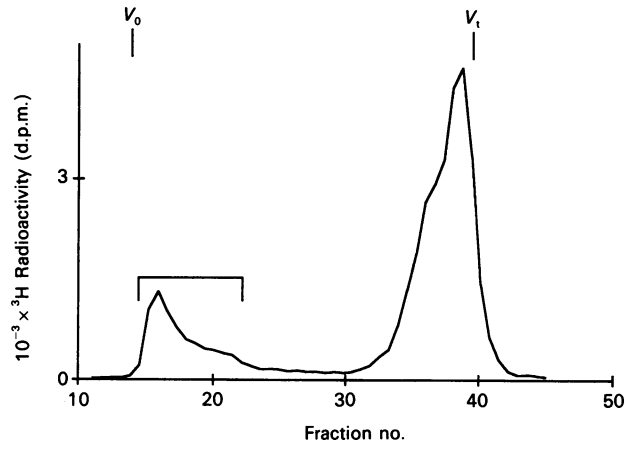


Fig. 1. Gel f.p.l.c. on Superose 6 of [^{3}H]glucosamine-labelled low-density HSPG after degradation with HNO_2

Purified low-density HSPG (from endothelial cell culture medium) was subjected to deaminative cleavage followed by gel chromatography. The column (HR 10/30; 25 ml) was equilibrated in 4 M-guanidinium chloride/0.1% (v/v) mulgophene/50 mM-sodium acetate, pH 5.8, and eluted with a flow of 0.4 ml/min using an Pharmacia-LKB f.p.l.c. system (Lindblom *et al.*, 1989). Samples of 200 μl were applied, and 78 s fractions were collected. Fractions containing core protein (with remnant *N*-acetylated segments) were pooled as indicated by the bar. The elution position of the intact proteoglycan is shown in Fig. 4.

from HSPG separated into a collection of bands where the dominant components co-migrated with DS oligosaccharides of d.p. approximately 10–17, even though some larger trailing oligosaccharides were also seen (Fig. 3*d*, lane 1). Caution must be taken in comparing the migration of DS and heparan oligosaccharides, since the presence of sulphate groups in the former should somewhat increase their migration. But on the whole, we think that the electrophoretic pattern of these reducing-end oligosaccharides indicates that they are polydisperse in size and that there exist small amounts of endothelial HS chains with very long (up to d.p. approx. 30) *N*-acetylated regions after the linkage tetrasaccharide, thus confirming the results of gel chromatography (Fig. 3*a* and *b*).

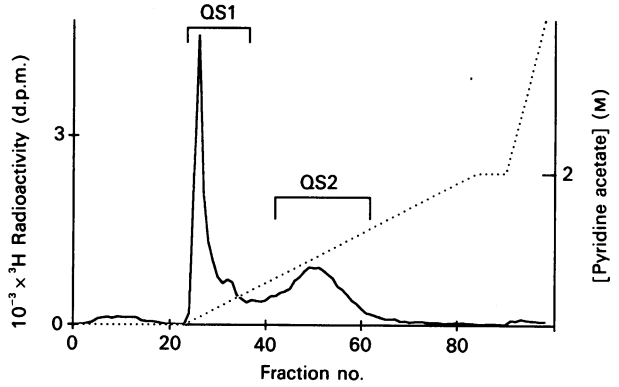


Fig. 2. Ion-exchange chromatography on Q-Sepharose Fast Flow of [^{3}H]glucosamine-labelled oligosaccharides released by alkaline elimination from the isolated HNO_2-degraded HSPG

HSPG-core protein, with the [^{3}H]glucosamine-labelled oligosaccharides remaining after deaminative cleavage, was pooled from Superose 6 chromatrography (Fig. 1) and subjected to alkaline elimination (see the Experimental section). After neutralization, the sample was partially desalted on a Fast Desalting column and freeze-dried. The sample was then redissolved in water and applied to the ion-exchange column (5 ml) equilibrated in 2 mM-pyridine acetate, pH 5.3. The column was eluted with sequential gradients of first 2 mM–2 M- and then 2–4 M-pyridine acetate, pH 5.3. The nominal gradient is indicated (...). Fractions were pooled (QS1 and QS2) as indicated by the bars. On Q-Sepharose Fast Flow, hyaluronic acid is eluted at approximately 3 M-pyridine acetate, whereas intact endothelial HS chains are not eluted with pyridine acetate but can be displaced by 2 M-NH_4HCO_3 (A. Lindblom, unpublished work).

Heparinase cleavage

Heparinase is an enzyme that specifically cleaves the hexosaminidic bonds of $GlcNSO_36R\alpha1$-4IdoA(2S) (Lindhardt *et al.*, 1990). This type of disaccharide is rare in human endothelial HS (approx. 6.5% of the disaccharides; Lindblom & Fransson, 1990), and digestion of the endothelial HSPG with heparinase therefore yields relatively few and large fragments. The progress and completion of the heparinase digestion was monitored as described in the Experimental section. As seen in Fig. 4, heparinase-digested HSPG was eluted bimodally on Superose 6, with approximately 40% of the [^{3}H]glucosamine label being eluted close to the void volume, whereas the rest was eluted at a more retarded position as a rather broad profile, representing oligosaccharides released from the HS chains.

The early peak from the Superose 6 chromatogram (Fig. 4), containing core protein with residual 'inner' fragments, was pooled and subjected to alkaline elimination. 'Outer' fragments were also pooled (OF in Fig. 4). Both 'inner' and 'outer' fragments comprised mostly higher-M_r oligosaccharides, as judged from their elution profile on TSK-Fractogel HW40 (results not shown; cf. Lindblom & Fransson, 1990) and were concentrated on DEAE-cellulose, freeze-dried and samples were subjected to gel chromography on Sepharose CL 6B (Fig. 5*a* and *b*). Both samples displayed broad elution profiles, indicating size heterogeneity, with peak maxima at $K_{av.}$ 0.5 and 0.65 for the 'inner' and 'outer' fragments respectively. Considering that the intact chains of the low-density HSPG were eluted at $K_{av.}$ 0.3 (as indicated in Fig. 5; c.f. Lindblom & Fransson, 1990), the results suggest that most chains, if not all, had been degraded by heparinase. When the isolated fragments were redigested with heparinase, their elution positions on Sepharose CL 6B were unchanged (not shown).

When treated with HNO_2 and chromatographed on Bio-Gel P6, the 'outer' fragments were more degraded that the 'inner'

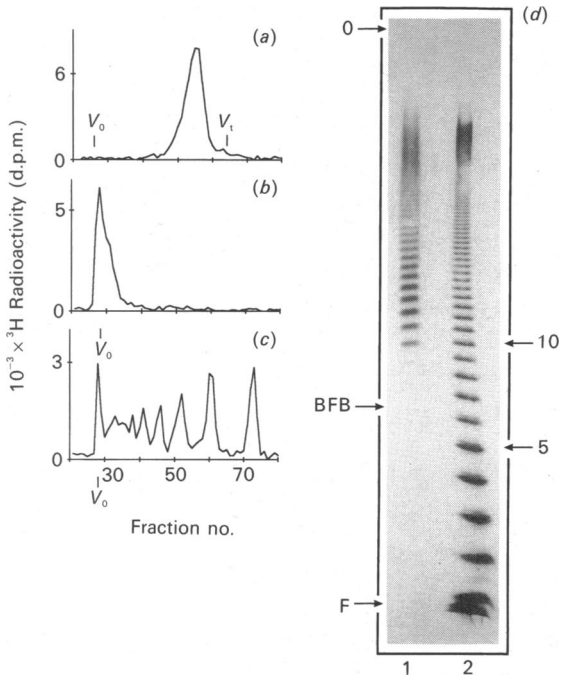


Fig. 3. Gel filtration and PAGE of polyanionic oligosaccharides released from the protein core after treatment with HNO_2

Oligosaccharide material, released by alkaline elimination (see the Experimental section) from the HNO_2-treated [^{3}H]glucosamine-labelled HSPG and eluted in the retarded peak (QS2) on ion-exchange chromatography (Fig. 2), was pooled and freeze-dried. Samples were then analysed by (*a*) gel chromatography on Sepharose CL 6B, (*b*) gel chromatography on Bio-Gel P6, (*c*) gel chromatography on Bio-Gel P6 after treatment with heparitinase. The Sepharose CL 6B column (1 cm × 100 cm) was equilibrated in 0.3 M-NaCl/10 mM-Tris/HCl, pH 7.5, and eluted at 4 ml/h. The Bio-Gel P6 column was equilibrated in 0.5 M-NH_4HCO_3 and was eluted at 8 ml/h. Fractions of 1.8 ml were collected. Void (V_0) and total volumes (V_t) were determined with Blue Dextran and free $^{35}SO_4^{2-}$ respectively. On Bio-Gel P6, disaccharides are eluted in fractions 71–74. In (*d*) is shown the gradient (20–30%) PAGE of oligosaccharides released by alkaline elimination from the HNO_2-treated HSPG and recovered by stepwise elution from DEAE-cellulose (see the Experimental section) and dialysis (Spectrapor 3). Lane 1, oligosaccharide generated by alkaline elimination of HNO_2-treated core protein; lane 2, endothelial DS (derived from fraction M 1b) treated with chondroitinase ACI. O, origin; BFB, migration of Bromophenol Blue on the same gel; F, migration front; 5 and 10 indicate the bands of DS oligosaccharide of d.p. 5 and 10.

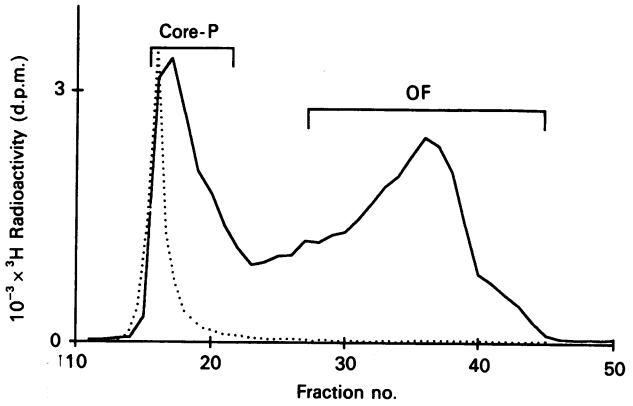


Fig. 4. Gel f.p.l.c. on Superose 6 of [^{3}H]glucosamine-labelled HSPG after digestion with heparinase

Purified low-density HSPG was digested as outlined in the Experimental section. The Superose 6 column was eluted as described in the legend to Fig. 1. The elution profiles of the intact (· · · ·) and digested (——) HSPG are shown. Fractions containing the early (denoted Core-P for core protein) and late peak (denoted OF for 'outer' fragments) were pooled as indicated.

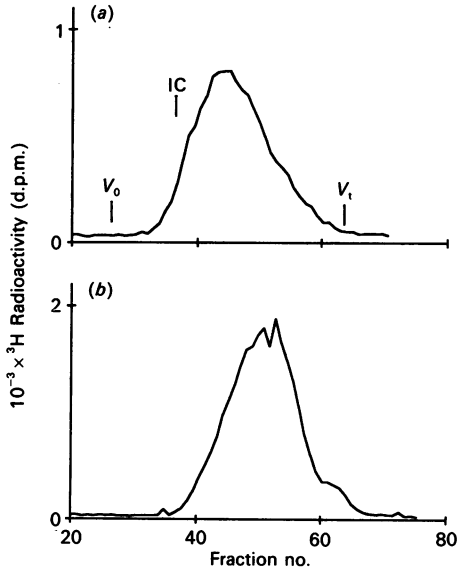


Fig. 5. Gel chromatography on Sepharose CL 6B of 'inner' and 'outer' oligosaccharide fragments obtained by heparinase digestion of [^{3}H]glucosamine-labelled HSPG

'Inner' (*a*) and 'outer' (*b*) fragments were obtained after heparinase digestion as described in the Experimental section. Samples were applied to the column and eluted as described in the legend to Fig. 3. V_0, void volume; V_t, total volume; IC, elution position of intact endothelial HS chains.

ones (Fig. 6*a*, and *b*), indicating a higher content of *N*-sulphamido groups in the 'outer' fragments. The HNO_2-treated heparinase fragments were also subjected to PAGE (Fig. 6*c*). Here the 'inner' fragments (lane 1) showed predominantly a population of larger oligosaccharides (d.p. approx. 6–20), although smaller saccharides were also seen. The 'outer' fragments (lane 2) yielded more of the smaller oligosaccharides but also contained extended *N*-acetylated regions (d.p. approx. 10–20). Large oligosaccharides (d.p. approx. 10–20) derived from 'inner' and 'outer' heparinase fragments had different banding patterns. This should be due to the presence of the linkage-region tetrasaccharide in oligosaccharides derived from 'inner' fragments, and perhaps also to the presence of ester sulphate in the oligosaccharides derived from the 'outer' fragments.

The 'inner' and 'outer' fragments were also examined by ion-exchange f.p.l.c. on Mono Q, and, as shown in Fig. 7, 'inner' fragments obtained after heparinase digestion could be further fractionated. At least two populations were resolved in the NaCl gradient, one minor (approx. 15% of the [^{3}H]glucosamine label in 'inner' fragments) eluted at approximately 0.2 M-NaCl (MQ1, Fig. 7*a*), and a second major peak eluted at approximately 0.65 M-NaCl (MQ2, Fig. 7*a*). 'Outer' heparinase fragments were eluted as one peak at 0.65 M-NaCl, although there was a minor shoulder of material eluted at lower NaCl concentrations (Fig. 7*b*).

The two populations obtained from 'inner' fragments were pooled and further analysed (results not shown). On Sepharose CL 6B, the material from the early peak (MQ1) was eluted as a homogeneous profile with $K_{av.}$ 0.75, which is similar to the elution position of the polyanionic oligosaccharides remaining

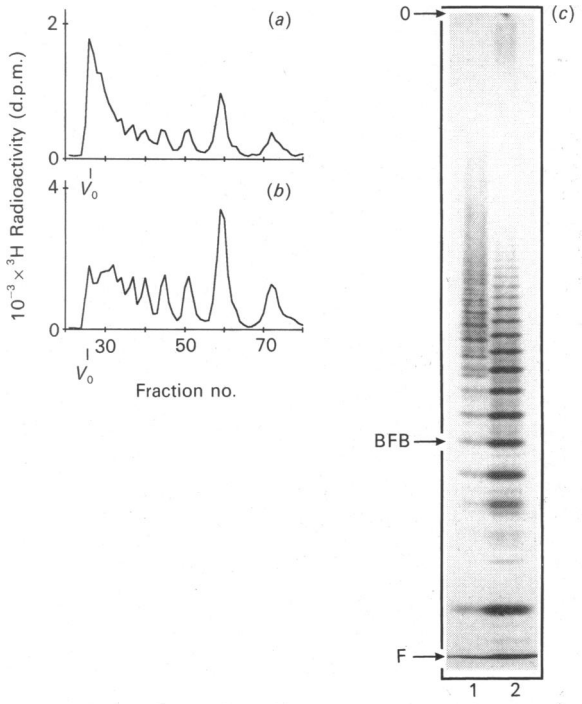


Fig. 6. Gel chromatography and PAGE of 'inner' and 'outer' fragments generated by heparinase digestion and further degraded by deaminative cleavage

Samples of the isolated 'inner' (*a*) or 'outer' (*b*) HS fragments were treated with HNO_2 and chromatographed on Bio-Gel P6. The column was eluted as described in the legend to Fig. 3. In (*c*) is shown the gradient (20–30%) PAGE of 'inner' (lane 1) and 'outer' (lane 2) fragments after deaminative cleavage. O, origin; BFB, Bromophenol Blue; F, migration front.

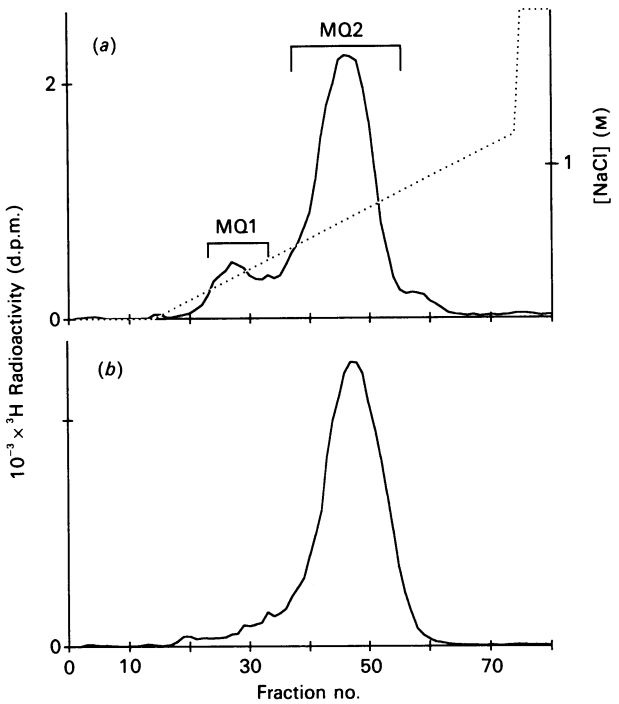


Fig. 7. Ion-exchange f.p.l.c. on Mono Q of 'inner' and 'outer' fragments obtained after heparinase digestion in low-density HSPG

'Inner' (*a*) and 'outer' (*b*) fragments were obtained after heparinase digestion of endothelial low-density HSPG as described in the Experimental section, and subjected to ion-exchange chromatography. A linear gradient of 0–1.2 M-NaCl was used and the nominal gradient is indicated in (*a*) (· · · ·). 'Inner' fragments were pooled into two populations (MQ1 and MQ2) as indicated in (*a*), and further analysed.

on the core protein after deaminative cleavage (Fig. 3*a*). Furthermore, the material eluted in the void volume on Bio-Gel P6 and the elution profile was unchanged after treatment with low-pH HNO_2, indicating that the material did not contain any *N*-sulphated disaccharides. The material from the late peak (MQ2) gave a more heterogeneous pattern with a peak maximum at $K_{av.}$ approximately 0.45. When the material in MQ2 was chromatographed on Bio-Gel P6 after deaminative cleavage, the degradative pattern was almost identical with that of the unfractionated 'inner' fragments after treatment with HNO_2 (results not shown, cf. Fig. 6*a*).

Affinity chromatography

When the intact endothelial HS chains, derived from the purified HSPG, were applied to the antithrombin III–Sepharose at low ionic strength, a major portion (65%) was bound to the column (Fig. 8*a*). Only minute amounts (< 1%), however, remained bound after elution with 0.2 M-NaCl and were displaced by 3 M-NaCl, where high-affinity heparin is eluted. In evaluating this low affinity of the chains towards antithrombin III, we also examined the relative affinity of 'inner' and 'outer' chain fragments generated by heparinase digestion. It appeared that these fragments had lost most of the low-affinity binding (see Fig. 8*b*,*c*), suggesting that this engages sites susceptible to heparinase in the native chains.

The HS chains from the endothelial HSPG all bound to lipoprotein lipase–agarose, with apparent affinities comparable with that of heparin. The peak of intact proteoglycans was eluted at a somewhat higher salt concentration than did the peak of free chains; 0.55 M-NaCl versus 0.48 M-NaCl (Fig. 9*a*). This may be explained by the possibility of multipoint attachment of one proteoglycan molecule to several lipase molecules, which would give a co-operative effect. The 'inner' and 'outer' fragments of HS chains were both eluted at lower salt concentrations than were the intact chains (around 0.25 M-NaCl, Fig. 9*b*).

General discussion

In the biosynthesis of heparin and HS, *N*-sulphation of GlcNAc residues constitutes a signal for further modifications of the same and adjacent saccharide residues, namely the 5′-epimerization of D-glucuronic acid (into L-iduronic acid) and the sulphation of hydroxy groups on C-3 and C-6 glucosamine and on C-2 of the uronic acid. Studies by Lindahl and co-workers [for references see Lindhal, (1989)] have shown that the modifications proceed in a certain order but are somewhat incomplete in the sense that not all potential sites are modified. Because of the many possible combinations of different saccharides, the end product is a very complex polysaccharide, with 18 different disaccharide constituents hitherto identified (Bienkowski & Conrad, 1985; Edge & Spiro, 1990).

Recent studies show that HS chains from different cells and tissues, even within one species, have distinctive characteristics, for example in polymer length, degree of *N*-sulphation and distribution and contents of various disaccharides (Pejler *et al.*, 1987; Horner, 1990; Turnbull & Gallagher, 1990; Lindblom & Fransson, 1990; Edge & Spiro, 1990). The observed differences may reflect unique combinations of the enzymes required for synthesis of HS in the different cells (Lindahl *et al.*, 1986). Also, the HS chains may be specific for different core proteins synthesized by the same cell.

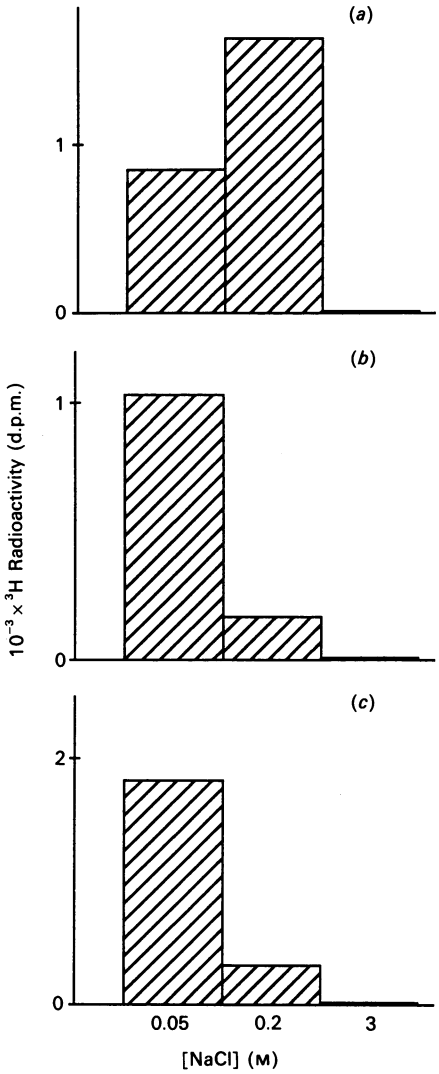


Fig. 8. Affinity chromatography on antithrombin III–Sepharose of endothelial HS

[^{3}H]Glucosamine-labelled intact HS chains and 'inner' and 'outer' heparinase fragments were isolated as described in the Experimental section. Samples, (150×10^3–250×10^3 d.p.m.) were dissolved in 500 μl of 50 mM-Tris/HCl, pH 7.5, and applied to the column, which was then eluted stepwise with (1) 50 mM-Tris/HCl, (2) 0.2 M-NaCl/50 mM-Tris/HCl, pH 7.5, and (3) 3 M-NaCl/50 mM-Tris/HCl, pH 7.5 (see the Experimental section). (*a*) Intact HS chains (derived from the low-density HSPG); (*b*) 'inner' fragments obtained after heparinase digestion as described; (*c*) 'outer' fragments.

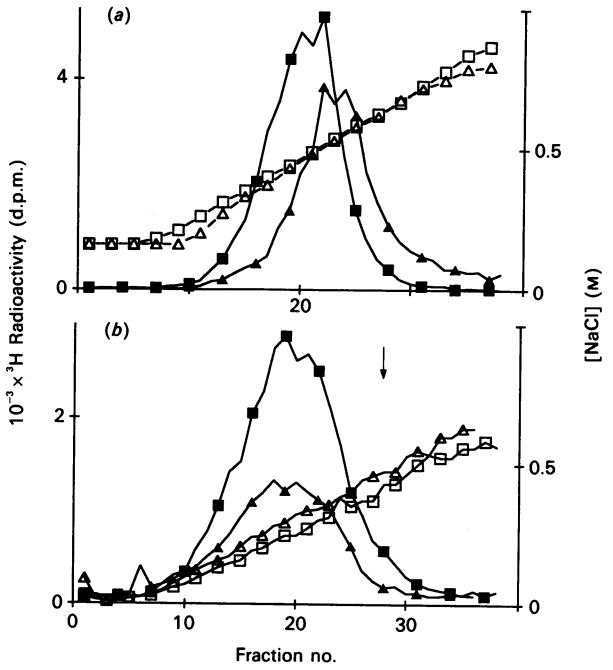


Fig. 9. Affinity chromatography on lipoprotein lipase–agarose of endothelial HS

The affinity gel was prepared and run as described (see the Experimental Section). (*a*) Elution profile for intact HSPG (▲), corresponding gradient (△). Native HS chains liberated from the low-density HSPG of the medium (■), corresponding gradient (□). The heparinase fragments applied in (*b*) were diluted in buffer without NaCl. 'Inner' fragments (▲), corresponding gradient (△), 'outer' fragments (■) and corresponding gradient (□). The arrow indicates the peak of the elution profile of intact HSPG run on the same batch of lipoprotein lipase–agarose, which was not the same as that used in (*a*).

The sequential distribution of *N*-acetylation and *N*-sulphation and also of other modifications in the chains is not fully understood but may be highly significant. For example, special saccharides have been proposed to provide a signal for termination of chain polymerization in the synthesis of chondroitin sulphate (Otsu *et al.*, 1985; Bourin *et al.*, 1990). Furthermore, the specific patterns of low and high sulphation might serve as important environmental signals in the extracellular matrix. Only a few studies with emphasis on the longitudinal distribution of the different disaccharides in HS chains have previously been published (Parthasarathy & Spiro 1984; Lyon *et al.*, 1987; Rosenfeld & Danishefsky, 1988; Edge & Spiro, 1990). In heparin already the second glucosamine residue appearing after the linkage tetrasaccharide was found to be *N*-sulphated (and hence sensitive to deaminative cleavage; Rosenfeld & Danishefsky, 1988), whereas a longer *N*-acetylated fragment, tentatively estimated as eight disaccharides, was identified in HS from human fibroblasts (Lyon *et al.*, 1987). A somewhat larger (and apparently less uniform) *N*-acetylated region was observed at the reducing end of chains derived from an HSPG of bovine glomeruli (Parthasarathy & Spiro, 1984).

Our results indicate that in human endothelial HS, the first *N*-sulphated glucosamine occurs at a variable position, approximately 10–17 disaccharides away from the protein–polysaccharide linkage. The electrophoretic pattern of these *N*-acetylated fragments further suggests that some may be larger than 20 disaccharides. Judging from the elution position of the isolated fragment on Sepharose CL 6B, the average size is approximately 12 disaccharides. Long *N*-acetylated regions (d.p. 10–20) were seen also in 'outer' fragments obtained by heparinase digestion and are thus not unique for the innermost parts of the HS chains. We could not detect any subpopulation of wholly unsulphated but fully polymerized chains, as reported by Iozzo (1989) for an HSPG from colonic carcinoma cells.

Endothelial HS had mostly solitary sites susceptible to heparin lyase (Lindblom & Fransson, 1990). When released from the protein core, 'inner' heparinase fragments were generally larger than 'outer' fragments. Both fragments are heterogeneous, indicating that the positioning of sulphated iduronic acid is not the same in all chains. 'Outer' fragments had a higher content of *N*-sulphated glucosamine (approx. 36% compared with approx. 28% in 'inner' fragments), as calculated from gel chromatographic profiles after deaminative cleavage (Fig. 3; cf. Lindblom & Fransson, 1990). On PAGE, the differences in composition of

the two fragments was further demonstrated by the different banding patterns of the large oligosaccharides (Fig. 3*c*). Finally, subfractionation of 'inner' heparinase fragments by ion-exchange f.p.l.c. indicated that in some chains an IdoA(2S) is situated immediately after the innermost *N*-sulphated glucosamine. In heparin chains, the average position of the first IdoA(2S) residue has been determined to be at the sixth disaccharide after the linkage tetrasaccharide (Rosenfeld & Danishefsky, 1988).

For a long time it has been believed that HS is present on the luminal surface of endothelial cells, providing 'heparin-like' cofactor activity to circulating antithrombin III (Damus *et al.*, 1973). Studies both *in situ* and *in vitro* have demonstrated a moderate antithrombin III cofactor activity, sensitive to heparin-degrading enzymes, in endothelium [for references see Marcum & Rosenberg (1987)]. However, little or no HSPG has been demonstrated at the luminal surface or even the plasma membrane of endothelial cells or vascular endothelium (Simonescu *et al.*, 1981), and the vascular binding-site of antithrombin III has recently been localized to the subendothelial matrix or basement membrane (de Agostini *et al.*, 1990). A high affinity for antithrombin III has earlier been demonstrated in the HS chains of a proteoglycan from the murine Reichert's membrane, a placental basement membrane structure (Pejler *et al.*, 1987). Endothelial HSPGs are also thought to serve as anchors for lipoprotein lipase, since this enzyme is released into the circulation on administration of heparin (Olivecrona & Bengtsson-Olivecrona, 1989).

The HSPG used in this study is the major HSPG present in cultures of endothelial cells from human umbilical vein, where it is present in both the medium and the cell layer. The core protein appears somewhat heterogeneous, perhaps due to proteolytic processing, but a major band with apparent M_r 350000 is seen on SDS/PAGE after digestion of the proteoglycan with heparitinase (Lindblom *et al.*, 1989). Only a minor amount of HSPG is released when the cell cultures are lysed with detergent, whereas the rest can be extracted with chaotropic buffers such as 6 M-guanidinium chloride, suggesting that the HSPG is located in the extracellular matrix.

Our results from affinity chromatography show that a majority of the chains of the endothelial HSPG bind to antithrombin III, but the affinity may be very low since it was broken by moderate salt concentrations. A specific pentasaccharide sequence has been identified in heparin with high affinity for antithrombin III (Thunberg *et al.*, 1982). On antithrombin–Sepharose, high-affinity heparin is not eluted at the concentrations used to elute endothelial HS, but, if the density of antithrombin-binding sites is lower in endothelial HS, the low M_r of heparin (10000–15000) as compared with endothelial HS (approx. 60000; Lindblom & Fransson, 1990) might explain part of this difference. Also, a low affinity might theoretically explain the binding and low-grade antithrombin III cofactor activity observed in the endothelium by Rosenberg and co-workers (Marcum & Rosenberg, 1987; de Agostini *et al.*, 1990). Final conclusions of whether endothelial HS has significant antithrombin III cofactor activity will have to await either direct measurements of its anticoagulant effects or demonstration of the presence or absence of the required pentasaccharide sequence within the chains.

With regard to lipoprotein lipase, the endothelial HS showed an affinity similar to that of heparin. As has been shown previously, the structural requirements for binding to lipoprotein lipase and antithrombin III are different. For example, whereas *N*-desulphation of heparin destroys all binding to both proteins, re-*N*-acetylation restores binding to lipoprotein lipase but not to antithrombin III (Bengtsson *et al.*, 1980). Heparin chains with high and low affinity for antithrombin III do not differ in binding to lipoprotein lipase (Bengtsson *et al.*, 1977). In the present study, 'inner' and 'outer' HS-fragments obtained by heparinase digestion showed similar affinity for lipoprotein lipase, although they showed a considerable difference in *N*-sulphate density. In contrast, the cleavage with heparinase destroyed almost all binding to antithrombin III.

In conclusion, we think our results confirm the idea that modifications along the polysaccharide chains display no absolutely constant features but rather a 'random' or 'probabilistic' variation around a certain 'average' structure. The polydisperse length of the innermost *N*-acetylated regions and the scattered occurrence of heparinase-sensitive sites [containing IdoA(2S)] agrees well with the proposed model for the biosynthesis of heparans, where the products are formed by a concerted but somewhat imprecise enzyme action (Lindahl *et al.*, 1986).

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