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Isolation and partial characterization of heparan sulphate proteoglycan from the human glomerular basement membrane

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Heparan sulphate proteoglycan was solubilized from human glomerular basement membranes by guanidine extraction and purified by ion-exchange chromatography and gel filtration. The yield of proteoglycan was approx. 2 mg/g of basement membrane. The glycoconjugate had an apparent molecular mass of 200–400 kDa and consisted of about 75% protein and 25% heparan sulphate. The amino acid composition was characterized by a high content of glycine, proline, alanine and glutamic acid. Hydrolysis with trifluoromethanesulphonic acid yielded core proteins of 160 and 110 kDa (and minor bands of 90 and 60 kDa). Alkaline $NaBH_4$ treatment of the proteoglycan released heparan sulphate chains with an average molecular mass of 18 kDa. HNO_2 oxidation of these chains yielded oligosaccharides of about 5 kDa, whereas heparitinase digestion resulted in a more complete degradation. The data suggest a clustering of *N*-sulphate groups in the peripheral regions of the glycosaminoglycan chains. A polyclonal antiserum raised against the intact proteoglycan showed reactivity against the core protein. It stained all basement membranes in an intense linear fashion in immunohistochemical studies on frozen kidney sections from man and various mammalian species.

INTRODUCTION

Heparan sulphate proteoglycans (HSPGs) are complex and heterogeneous macromolecules composed of linear sulphated polysaccharide chains, the heparan sulphate moieties, that are covalently attached to protein [1–3]. HSPGs have been classified into at least two types, which differ with respect to their distribution and functions [3–7]. The cell-associated HSPGs have been strongly implicated in cell adhesion processes [8–10]. The basement-membrane forms, notably those found in the glomerular basement membrane (GBM), are thought to control macromolecular permeability on the basis of their anionic charge [11].

HSPGs have been isolated and characterized from rat, mouse, human and horse glomeruli [12–18] and from GBM of rat, cattle and man [19–21]. These proteoglycans vary considerably in size and density. Many studies have relied to a great extent on metabolic radiolabelling [12–14,19]. Differences in turnover may, however, cause variations between glycosaminoglycan composition found by isolation and observed after radiolabelling [22–24]. The variations might also be related to the presence of different HSPG populations in glomeruli and GBM [25], although Kanwar *et al.* [14] described the presence of the same type of HSPG in rat glomeruli and GBM. In agreement with labelling studies in the rat [24,25], we found that less than 20% of the glomerular glycosaminoglycan content of man and horse was recovered in GBM preparations [18].

Most immunohistochemical studies have been performed with antibodies against HSPG from EHS tumour [26]. Native basement membranes contain components similar to, at least at the immunological level, those in the EHS tumour [27,28]. However, HSPGs isolated from glomerular and Reichert's basement membranes [20,29,30] are clearly different from those isolated from basement-membrane material produced by tumour cell lines with respect to size of core protein and degree of sulphation [24,31–33].

Previously, we determined the glycosaminoglycan content of glomerular and tubular basement membranes of various mammalian species [34] and of human individuals of different ages [35]. The procedure was also adequate for application on basement membranes isolated from patient kidneys after autopsy (results not published). The availability of specific polyclonal and monoclonal antibodies against human GBM HSPG is essential, however, to establish the involvement of these compounds in studies of renal pathology on biopsies. Shimomura & Spiro [21] recently described the characterization of HSPG and its core protein from human GBM on the basis of radiolabelling and immunochemical reaction with anti-(bovine GBM HSPG) antibodies. In the present paper we describe the isolation and characterization of four HSPG preparations from human GBM by a procedure that avoids proteolytic interference as far as possible. We show that the structure of this compound is largely different from that isolated from glomeruli on the basis of both chemical and immunochemical data. We prepared for the first time specific polyclonal antibodies against human GBM HSPG and used them for immunohistochemical studies.

Abbreviations used: EHS, Engelbreth–Holm–Swarm; GBM, glomerular basement membrane; HS, heparan sulphate; HSPG, heparan sulphate proteoglycan; TFMS, trifluoromethanesulphonic acid.

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MATERIALS AND METHODS

Materials

Intestinal mucosal heparin, laminin (EHS sarcoma), type IV collagen (human placenta), fibronectin (human serum), albumin (human serum), ferritin, thyroglobulin. Alcian Blue and *o*-phenylenediamine were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Anisole, trifluoromethanesulphonic acid (TFMS) and Toluidine Blue were purchased from Merck, Darmstadt, Germany. DEAE-Sepharose CL-6B (fast flow), Sepharose CL-4B, Sephacryl S200 and Sephadex G-100 were from Pharmacia, Uppsala, Sweden. 4-Chloro-1-naphthol was from Janssen Chimica, Beerse, Belgium. High-molecular-mass markers, Bio-Gel P-2 and Bio-Gel P-30 were from Bio-Rad Laboratories, Richmond, CA, U.S.A. Peroxidase-conjugated pig anti-(rabbit IgG) serum was from DAKO Immunoglobulins, Glostrup, Denmark. Peroxidase-conjugated goat anti-(rabbit IgG) serum and goat anti-(rabbit IgG–fluorescein isothiocyanate) serum were from Nordic, Tilburg, The Netherlands. NaB^3H_4 (21.6 Ci/mmol) was from Amersham International, Amersham, Bucks., U.K. Pig mucosa heparin standard, polyclonal antibody (rabbit) against human placenta laminin and monoclonal antibody (mouse) against human laminin type I were from Calbiochem, San Diego, CA, U.S.A. Polystyrene micro-titre plates were from Greiner, Alphen aan de Rijn, The Netherlands. All other chemicals were as described elsewhere [18] or of analytical grade.

Preparation of glomerular basement membranes (GBM)

Human kidneys were obtained at autopsy within 20 h after natural death and stored for up to 6 months at −20 °C. During their life these persons (both sexes, aged 20–85 years) were free from clinical symptoms of any renal disease and the kidneys did not show any macroscopic lesions. Glomeruli were isolated by a graded sieving procedure [35]. Subsequently GBM was prepared by a detergent procedure in the presence of proteinase inhibitors (1 mM-phenylmethanesulphonyl fluoride, 5 mM-benzamidine hydrochloride, 10 mM-*N*-ethylmaleimide, 0.1 M-6-aminohexanoic acid, 5 mM-iodoacetamide and 10 mM-EDTA) [18,36].

Extraction of proteoglycan from GBM

All procedures were carried out at 4 °C and in the presence of the mixture of proteinase inhibitors. An amount of about 7.5 g of basement membrane (originating from about 120–140 adult kidneys) was extracted twice with 1.5 litres of 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8 (buffer A), with stirring for 16 h at 4 °C. The combined supernatants (100000 ***g*** for 30 min) were concentrated by ultrafiltration on an Amicon PM 10 filter and extensively dialysed against 7 M urea/10 mM-Tris/HCl buffer, pH 6.8.

Purification of HSPG by DEAE-Sepharose chromatography and gel filtration

Samples dialysed into urea were chromatographed, after centrifugation (100000 ***g*** for 30 min), on a column (5.0 cm × 25.0 cm) of DEAE-Sepharose CL-6B (fast flow) equilibrated with 7 M-urea/10 mM-Tris/HCl buffer, pH 6.8, as previously described [18]. The glycosaminoglycan content of the fractions was determined by the Farndale assay [37]. Fractions containing the glycosaminoglycan were pooled, concentrated (by filtration on an Amicon PM 10 filter) and extensively dialysed against 7 M-urea/10 mM-Tris/HCl buffer, pH 6.8. The material was submitted to chromatography on a second DEAE-Sepharose column (5.0 cm × 5.0 cm) and eluted as before. The proteoglycan fractions were dialysed against buffer A and loaded on a Sepharose CL-4B column (1.6 cm × 108 cm) equilibrated and eluted with buffer A. Finally the concentrated proteoglycan sample was loaded on a Sephacryl S200 column (2.6 cm × 87.5 cm), which was eluted again with buffer A. The HSPG-containing fractions were pooled, dialysed against distilled water and freeze-dried.

Identification of proteoglycans

Proteoglycans were identified by treatment with heparitinase and chondroitin ABC lyase according to previously described procedures [18,34].

Isolation and characterization of glycosaminoglycan chains

Samples of HSPG (365 μg) were incubated in 150 μl of 0.1 M-NaOH containing 0.1 M-NaB^3H_4 (167 μCi/μmol) at 37 °C for 72 h [20]. The released [^{3}H]glycosaminoglycan chains were purified by chromatography on a Bio-Gel P-2 column (1.6 cm × 75 cm) and a DEAE-Sepharose column (1.0 cm × 10 cm) as described by Parthasarathy & Tanzer [38].

The molecular mass of the [^{3}H]glycosaminoglycan chains was determined by chromatography on a Sephadex G-100 column (1.6 cm × 72 cm) according to the procedure of Wasteson [39]. To permit further characterization, the purified glycosaminoglycans were chromatographed on a column (1.6 cm × 150 cm) of Bio-Gel P-30 (100–200 mesh) equilibrated and eluted with buffer A. The procedure was also performed after HNO_2 and heparitinase treatment. Portions of [^{3}H]glycosaminoglycan were treated with 150 μl of 0.50 M-HNO_2 [40] for 80 min at room temperature or with heparitinase (1 unit/ml) at 43 °C for 16 h in 0.1 M-sodium acetate/10 mM-calcium acetate buffer, pH 7.0.

Preparation of core protein

The core protein of the proteoglycan was prepared by using TFMS hydrolysis in accordance with Edge *et al.* [41]. To isolate the deglycosylated protein the reaction mixture was diluted with 2 vol. of diethyl ether cooled to −70 °C. After addition of an equal volume of ice-cold aq. 50% (v/v) pyridine the resulting precipitate was redissolved by vortex-mixing the suspension and the ether phase was removed. After a second diethyl ether extraction the aqueous solution was dialysed against water and freeze-dried.

Preparation and characterization of rabbit antibodies

Rabbit antiserum against intact HSPG from human GBM was prepared either by a schedule of multiple intradermal injections [42] or according to the schedule of Louvard *et al.* [43]. Specificity and titre of the antisera were determined by e.l.i.s.a. [44,45].

Polyacrylamide-gel electrophoresis

HSPG or its core protein was run into an SDS/3–20% polyacrylamide gel overnight at 60 V according to the procedure of Laemmli [46]. Staining was accomplished with Coomassie Blue [47], silver [48], Alcian Blue [47] or

periodic acid/Schiff reagent [49]. High-molecular-mass standards (Bio-Rad Laboratories) and laminin were used as molecular-mass markers.

Immunoblotting and staining

Electrophoretic transfer of antigens, separated by SDS/polyacrylamide-gel electrophoresis, on to unmodified sheets of nitrocellulose was carried out as described by Towbin *et al.* [50] and Burnette [51]. Transfer was achieved at 250 mA overnight with constant stirring. Molecular-mass standard proteins were detected by staining with Coomassie Blue. After blotting, the nitrocellulose filter was blocked by incubation for 1 h with 0.35 M-NaCl/10 mM-Tris/HCl buffer, pH 7.6, containing 3% (w/v) bovine serum albumin. Incubations with the anti-HSPG serum were performed for 2 h with a 1:100 to 1:500 dilution with 0.15 M-NaCl/10 mM-Tris/HCl buffer, pH 7.6, containing 0.3% bovine serum albumin, 1.0% (v/v) Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS. Pre-immune serum (diluted 1:100 in the same buffer) was used as a control. Antibody binding was detected with peroxidase-conjugated anti-(rabbit IgG) serum by using the method of Hawkes *et al.* [52].

Fluorescence microscopy

Indirect immunofluorescence microscopy with the anti-HSPG serum was carried out as described by Aufderheide *et al.* [53] with human and mammalian kidneys.

Analytical procedures

Protein, uronic acid and glycosaminoglycan contents, as well as amino acid composition, were determined according to previously described procedures [18].

RESULTS

Isolation of proteoglycan from human GBM

After two extractions of GBM with 4 M-guanidine 54 ± 12% (mean ± S.D., four preparations) of the glycosaminoglycan content of the GBM was solubilized. A comparable extraction efficiency was reported for rat GBM [19]. When the guanidine extract of the basement membranes, after dialysis against 7 M-urea, was chromatographed on DEAE-Sepharose, all the material, positive in the Farndale assay, was bound to the column and was eluted as a broad peak between 0.5 M- and 0.8 M-NaCl (Fig. 1). Rechromatography on DEAE-Sepharose yielded an additional separation of contaminating protein from the proteoglycan peak.

Upon chromatography of this preparation on Sepharose CL-4B in 4 M-guanidine the Farndale-positive material emerged in a position between 330 kDa ($K_{av.}$ 0.37) and 220 kDa ($K_{av.}$ 0.80) (Fig. 2). The same material contained uronic acids and protein (results not shown). Additional gel filtration on Sephacryl S200 was necessary to remove some low-molecular-mass proteins.

Composition of the proteoglycan

During the purification the proteoglycan material obtained at each step was analysed for its content of protein and glycosaminoglycan (Table 1). Analysis of the four final proteoglycan preparations indicated that peptide and glycosaminoglycan accounted for about 75% and 25% respectively of its residue weight. The yield was approx. 2 mg/g of GBM, which is equivalent to about 1–2 mg/kg of kidney cortex. The nature of the glycosaminoglycan side chains was determined by enzymic digestion. About 85% of the Farndale-positive material was sensitive to heparitinase, whereas chrondroitin ABC lyase treatment had barely any effect. This indicated the absence of chondroitin sulphates and dermatan sulphate. The HSPG preparations did not react in e.l.i.s.a. with polyclonal antibodies against mouse laminin, which reacted well with human kidney in immunofluorescence, and also did not react with a polyclonal antibody against human collagen IV and a monoclonal antibody against human laminin.

The amino acid composition of the HSPG was notable for the high content of glutamic acid (+glutamine), glycine, alanine and proline (Table 2). Also, the values of serine and aspartic acid are rather high. Cysteine and methionine could be detected only in minor amounts. 3-Hydroxyproline, 4-hydroxyproline and hydroxylysine are lacking, which establishes the absence of collagenous material. In all preparations glucosamine represents 90–95% of the amino sugars. In accordance with the

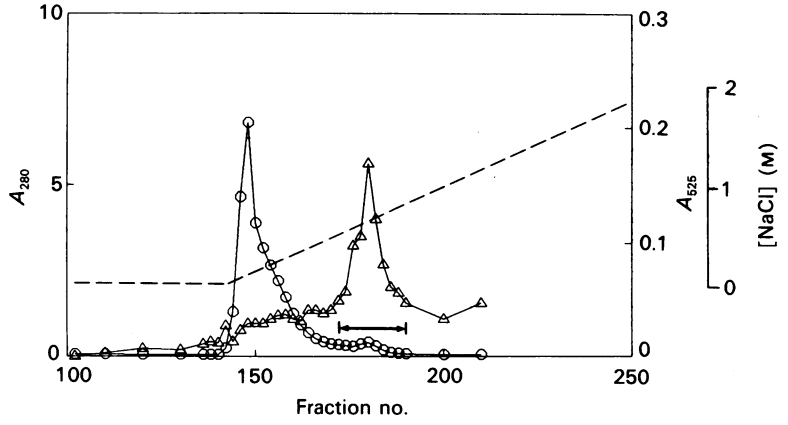

Fig. 1. Chromatography of a human GBM extract on DEAE-Sepharose CL-GB (fast flow)

Elution was with 7 M-urea/10 mM-Tris/HCl buffer, pH 6.8, followed by a gradient of 0–2 M-NaCl (——) in the same buffer. Fractions (8 ml) were analysed for protein (A_{280}) (○) and glycosaminoglycan (A_{525}) (△). Fractions 170–190 were pooled for further purification.

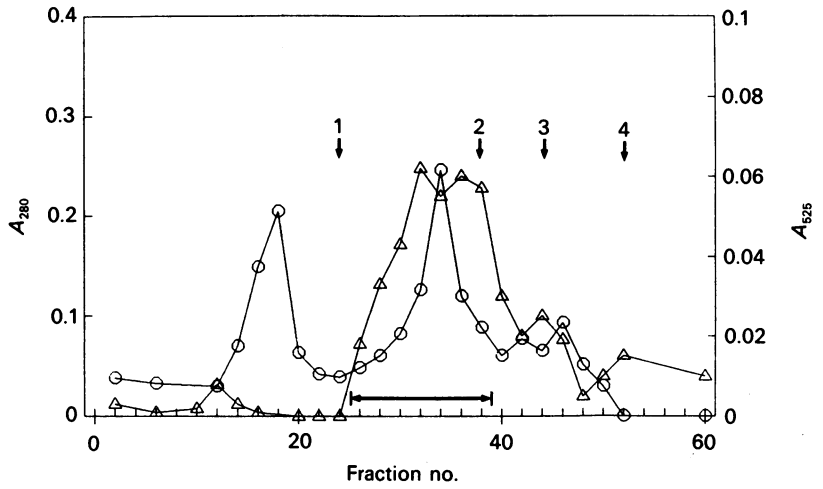

Fig. 2. Gel chromatography on Sepharose CL-4B of human GBM proteoglycan obtained after second DEAE-Sepharose chromatography

The column was eluted with buffer A. Fractions (5 ml) were analysed for protein (A_{280}) (○) and glycosaminoglycan (A_{525}) (△). The elution positions of thyroglobulin (330 kDa), ferritin (220 kDa), albumin (67 kDa) and cytochrome *c* (12 kDa) are indicated by arrows 1–4 respectively. Fractions 25–39 were pooled for further purification.

Table 1. Purification of HSPG from human GBM

Experimental details are given in the Materials and methods section. Values are given as means ± S.D. for four preparations.

Purification step	Protein (mg/g of basement membrane)	Glycosaminoglycan (mg/g of basement membrane)	Protein/glycosaminoglycan ratio
Guanidine extraction	313±33	1.78±0.45	194±17
Urea extraction	128±108	0.80±0.39	145±54
First DEAE-Sepharose chromatography	7.34±7.84	0.71±0.37	8.57±5.13
Second DEAE-Sepharose chromatography	3.66±3.63	0.62±0.36	6.44±2.34
Sepharose CL-4B chromatography	23.18±1.81	0.51±0.26	3.91±1.17
Sephacryl S200 chromatography	1.57±1.26	0.47±0.27	3.20±0.72

enzymic digestions this indicates the presence of only heparan sulphate as glycosaminoglycan. The trace of galactosamine could have originated from oligosaccharide chains. Preliminary analyses by g.l.c. showed the presence of small amounts of galactose, xylose and mannose.

Characterization of the HSPG and its core protein

SDS/polyacrylamide-gel electrophoresis of HSPG revealed after silver staining one major broad band with an apparent molecular mass of 200–400 kDa (Fig. 3). The same band could likewise be detected by staining the gels with Alcian Blue, Toluidine Blue, periodic acid/Schiff reagent or Coomassie Blue (results not shown).

Treatment of the proteoglycan with TFMS, a reagent that cleaves all saccharide units, resulted in a decrease in its apparent molecular mass to main bands of 160 and 110 kDa and a minor band of 60 kDa (Fig. 4). The pronounced sharpening of the electrophoretic bands after the TFMS treatment indicates that the appearance of the native proteoglycan as a broad band on the polyacrylamide gel is mainly due to variability in its saccharide chains. HNO_2 or heparitinase treatment of the HSPG resulted in a less substantial decrease in molecular mass than TFMS hydrolysis, but the proteoglycan band disappeared completely (results not shown). These results were obtained with all four preparations.

Characterization of the glycosaminoglycan chains

After β-elimination with alkaline NaB^3H_4 the glycosaminoglycans ^3H-labelled at the xylitol end were purified by Bio-Gel P-2 chromatography and subsequently DEAE-Sepharose chromatography to remove labelled peptide and oligosaccharide fragments. Their molecular mass was determined by gel filtration on Sephadex G-100 (Fig. 5). They were eluted as a broad peak at a $K_{av.}$ of 0.19, indicating a molecular mass of about 18 kDa. More than 90% of the glycosaminoglycans was HS according to their sensitivity to heparitinase.

The glycosaminoglycans emerged from a Bio-Gel P-30 column as a symmetrical peak ($K_{av.}$ 0.04) consistent with the molecular mass of 18 kDa (Fig. 6). The radiolabelled products of the deaminative degradation with HNO_2 were of substantially smaller size, and encom-

Table 2. Amino acid composition of HSPG preparations of human GBM

Values are given as means ± S.D. for four preparations.

Amino acid or amino sugar	Composition (residues/1000 amino acid residues)
Aspartic acid + asparagine	87 ± 4
Threonine	54 ± 3
Serine	84 ± 4
Glutamic acid + glutamine	140 ± 8
Proline	89 ± 5
Glycine	141 ± 13
Alanine	99 ± 3
Cysteine	5 ± 1
Valine	67 ± 2
Methionine	7 ± 2
Isoleucine	23 ± 3
Leucine	68 ± 3
Tyrosine	20 ± 0
Phenylalanine	25 ± 1
Histidine	17 ± 1
Lysine	26 ± 3
Arginine	58 ± 11
Glucosamine	127 ± 26
Galactosamine	9 ± 11

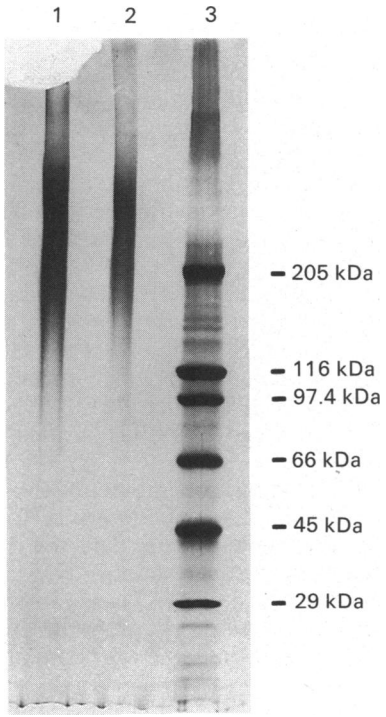

Fig. 3. SDS/polyacrylamide-gel electrophoresis of human GBM HSPG in 3–20% polyacrylamide slab gel

Per track 5.0 μg (track 1) or 1.0 μg (track 2) of HSPG was applied and the gel was stained with silver. Track 3, markers.

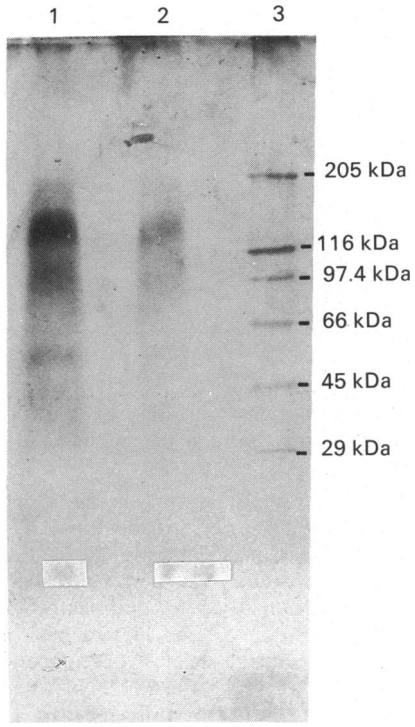

Fig. 4. SDS/polyacrylamide-gel electrophoresis of the core protein (obtained after TFMS treatment) of human GBM HSPG in 3–20% polyacrylamide slab gel

Per track 10 μg (track 1) or 2 μg (track 2) of core protein was applied and the gel was stained with silver. Track 3, markers.

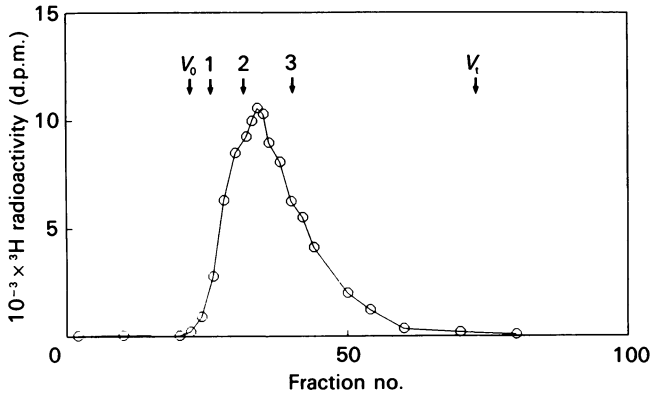

Fig. 5. Gel chromatography on Sephadex G-100 of the [^{3}H]-glycosaminoglycan chains obtained by alkaline NaB^{3}H$_4$ treatment of HSPG

The [^{3}H]glycosaminoglycan chains obtained by alkaline NaB^{3}H$_4$ treatment of HSPG and purified by Bio-Gel P-2 and DEAE-Sepharose chromatography were applied to a column equilibrated with 0.2 M-NaCl. After elution with the same solution each fraction was analysed for radioactivity. The void volume (V_0) and the total volume (V_t) of the column are indicated, and the elution positions of reference 26.0 kDa, 21.0 kDa and 14.3 kDa glycosaminoglycans are indicated by arrows 1–3 respectively.

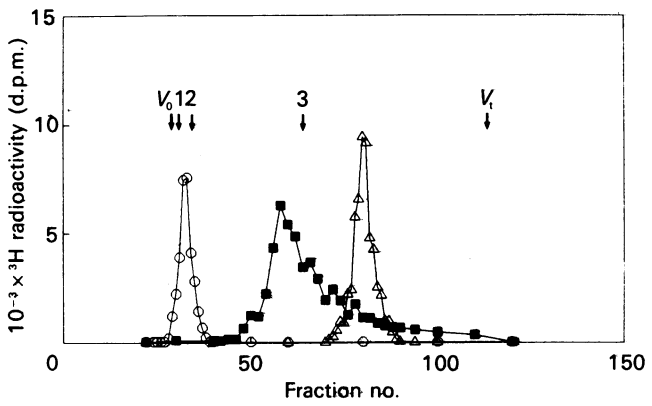

Fig. 6. Gel chromatography on Bio-Gel P-30 of intact and degraded [^{3}H]glycosaminoglycan chains of human GBM HSPG

○, Intact [^{3}H]glycosaminoglycan chains of human GBM HSPG. Degradation was performed with HNO_2 treatment (■) and with heparitinase digestion (△). Equilibration and elution of the column was performed with buffer A. Each fraction was analysed for radioactivity. The void volume (V_0) and total volume (V_t) of the column are indicated, and the elution positions of intestinal mucosa heparin (21.0 kDa), cytochrome *c* (12.4 kDa) and adrenocorticotropin (3.5 kDa) are indicated by arrows 1–3 respectively.

passed a broader molecular-mass range (average 5 kDa). Large segments of the internal portion of glycosaminoglycan chains are apparently devoid of *N*-sulphate groups and are therefore resistant to HNO_2. Digestion of the glycosaminoglycan chains with heparitinase effected a more complete degradation to the short stubs from the reducing end ($K_{av.}$ 0.60).

Characterization of the anti-HSPG serum

Polyclonal antibodies were raised against the proteoglycan in rabbits according to two immunization schedules. Both protocols resulted in antisera of comparable titres. The schedule of Vaitukaitis [42] needed, however, less antigen (approx. 100 μg instead of 500 μg). By e.l.i.s.a., no antibody activity could be detected against mouse laminin (EHS-tumour-derived) and human type IV collagen, albumin or fibronectin. The polyclonal antibodies showed no reactivity against bovine kidney HS, to the Pronase digest of HSPG or to HS released by treatment with alkaline $NaBH_4$. The antibodies showed a clear reactivity against the core protein (obtained after heparitinase or TFMS treatment of the HSPG) (Fig. 7). The antibodies seem to recognize specifically epitopes present on GBM HSPG, since we observed only a slight reactivity (< 5%) with HSPG purified from human glomeruli [18].

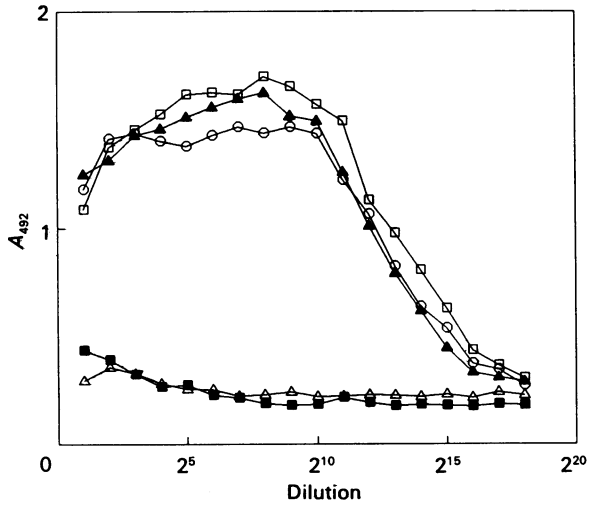

Fig. 7. E.l.i.s.a. with the anti-HSPG serum against intact HSPG (1 μg/well) (○), its core protein obtained after TFMS hydrolysis (1 μg/well) (▲), heparitinase-digested HSPG (1 μg/well) (□), Pronase-digested HSPG (1 μg/well (■) and HS (5 μg/well) (△)

Pre-immune serum gave no reaction (result not shown).

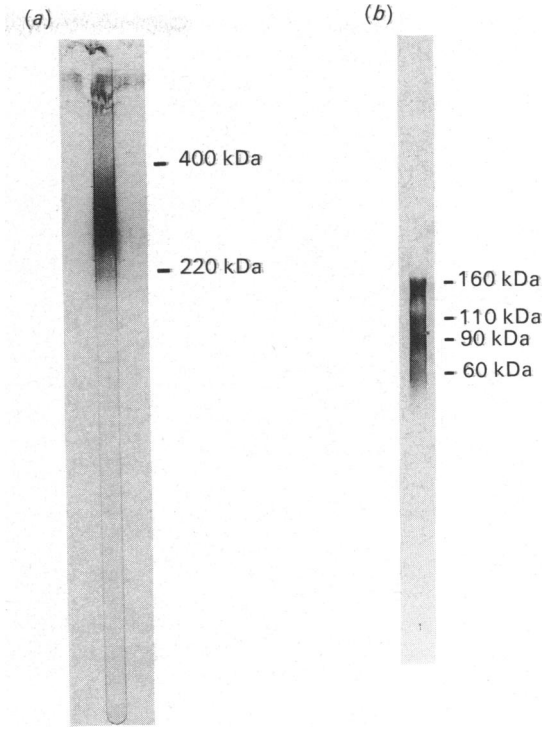

Fig. 8. Western blots of intact human GBM HSPG (*a*) and its core protein obtained by TFMS treatment (*b*) subjected to electrophoresis on SDS/3–20% polyacrylamide gels

Immunostaining was performed with anti-HSPG serum and peroxidase-conjugated goat anti-(rabbit IgG) antibodies [51].

On immunoblotting, the antiserum showed a positive binding to both the native HSPG as well as to the core protein (Fig. 8). The antiserum detected the same bands as those detected by silver staining (Figs. 3 and 4). An additional core protein of 90 kDa became visible. Seventeen monoclonal antibodies prepared against human GBM HSPG showed the same four core proteins (results not shown).

In immunofluorescence studies the antibodies specifically stained basement membranes of human kidney (Fig. 9) in an intense linear pattern, not only the glomerular basement membrane, but also tubular and peritubular capillary basement membranes and Bowman's capsule. The antiserum showed the same reaction pattern with horse, rat, mouse and hamster kidney (results not shown).

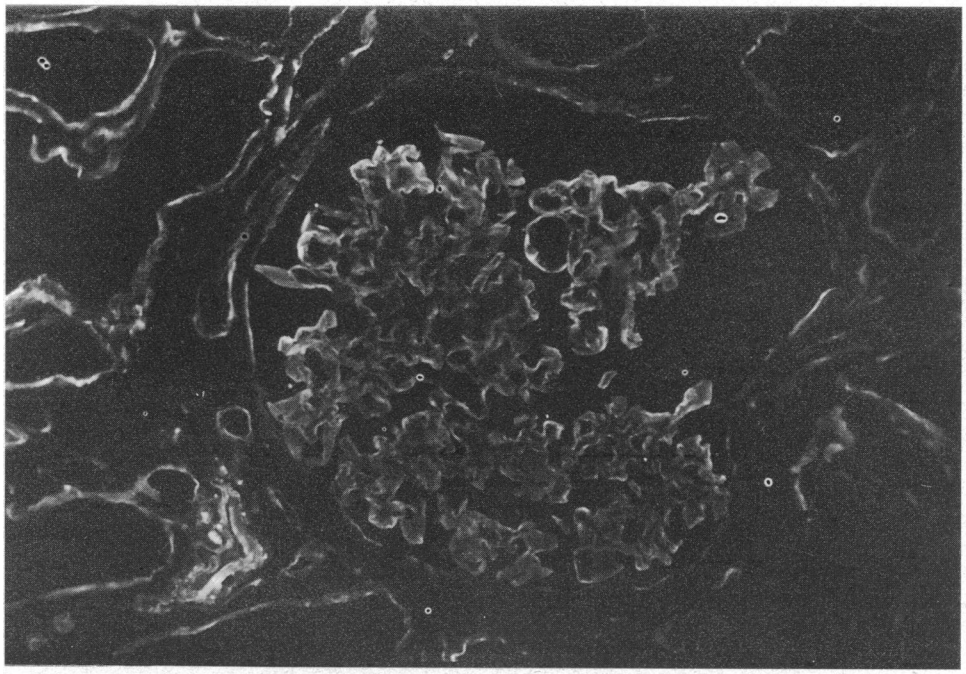

Fig. 9. Immunofluorescent staining of human kidney cortex with anti-(human GBM HSPG) serum (dilution 1:500)

Magnification ×80.

DISCUSSION

We isolated HSPG from the human GBM by guanidine extraction, repeated ion-exchange chromatography and subsequent gel filtration on Sepharose CL-4B and Sephacryl S200. This procedure was chosen to avoid the use of chondroitin lyases, since these treatments may lead to proteolytic degradation by contaminating proteinases. Proteolytic degradation during isolation of glomeruli, GBM and HSPG was prevented as far as possible by performing the procedures at 4 °C and adding a mixture of proteinase inhibitors. The HSPG content of GBM is low (about 1%), but the recovery after isolation was relatively good (10–30%). The isolated proteoglycan preparations were sensitive to heparitinase and resistant to chondroitin ABC lyase, and had a molecular mass of 200–400 kDa. Previous estimations of the molecular mass of GBM HSPG vary from 130–185 kDa for rat GBM [12,19], to 200 kDa for bovine GBM [54] and to 350 and 210 kDa (two forms) for human GBM [21]. The last-mentioned values are comparable with our data.

The molecular mass of 18 kDa for the HS chains is in the range of these polymers from rat GBM (26 kDa) [19] and bovine GBM (14 kDa) [20]. The HNO_2 degradation of the ^{3}H-labelled HS chains indicated an uneven distribution of *N*-sulphate groups along the HS chains. As for bovine GBM HSPG [20], we found for human GBM GSPG that the *N*-sulphated groups are preferentially located in the distal region of the HS chains. An extended *N*-acetylated sequence adjacent to the protein-linkage region was also found in human skin fibroblast HSPG [55]. Whether this feature has functional implications needs further investigation.

The presence of more core proteins after TFMS treatment of HSPG may be due either to proteolytic processing of a precursor protein or to proteolysis occurring after death, during storage of kidneys, glomeruli and GBM preparations or during the extensive purification procedures. The presence of the same core proteins in all four HSPG preparations and their similar reaction with polyclonal and monoclonal antibodies provide evidence for their relation with HSPG. Our data for the main core proteins of human GBM HSPG liberated by TFMS are in agreement with those reported by Shimomura & Spiro [21]. Bovine GBM HSPG gave only one core protein of 128 kDa (after TFMS treatment) [54]. The value for the core protein of HSPG of rat GBM was markedly lower [19]. Variations might be related to interspecies differences, but proteolysis is probably responsible for the small size of the core protein from rat GBM HSPG, as observed by Kanwar [19], since recently [56] the presence of a 130 kDa core protein in rat GBM HSPG was reported. Antibodies against EHS-tumour HSPG detected in extracts of rat GBM, after treatment with heparitinase, core proteins with apparent molecular masses of 250, 200 and 150 kDa [16].

The protein content of HSPG from human GBM is comparable with that from bovine GBM [20], but clearly aberrant from that of rat GBM [19]. Amino acids typical of collagen IV were lacking in our preparations and HSPG from rat GBM [19], but present in that from bovine GBM [20]. A high number of (half-)cystine residues was found for bovine GBM HSPG, but this amino acid was barely detectable in HSPG from human and rat GBM. On the basis of the protein content and the apparent molecular masses of the intact HSPG, the core protein (after TFMS treatment) and the HS chains, human GBM HSPG may contain three or four HS side chains and have a structure comparable with that suggested for HSPG from bovine GBM [57].

In e.l.i.s.a. the polyclonal anti-(GBM HSPG) serum

showed little reaction with HSPG preparations isolated from human glomeruli [18]. These preparations contained, however, only 40% protein and had clearly a different amino acid composition. The occurrence of different HSPG populations in glomeruli [24] and the low proportion of the glomerular glycosaminoglycan content present in the GBM [18] may explain these differences, but it may be possible that proteolytic degradation has taken place during isolation of glomerular HSPG.

Immunoblotting studies and e.l.i.s.a. confirmed that the polyclonal antibodies to the human GBM HSPG did not react with other basement-membrane components or albumin. In addition, the antiserum did not react with the glycosaminoglycan portion. Therefore the antiserum probably recognizes epitopes on the core protein of the proteoglycan, although in addition it may possess reactivity against *N*- or *O*-linked oligosaccharides. The immunohistochemical study demonstrated staining of renal basement membranes of human and various other mammalian species in an intense linear pattern.

REFERENCES

1. Lindahl, U. & Höök, M. (1978) Annu. Rev. Biochem. **47**, 385–417
2. Rodén, L. (1980) in The Biochemistry of Glycoproteins and Proteoglycans (Lennarz, W. J., ed.), pp. 267–371, Plenum Publishing Corp., New York
3. Gallagher, J. T., Lyon, M. & Steward, W. P. (1986) Biochem. J. **236**, 313–325
4. Stow, J. L., Sawada, H. & Farquhar, M. G. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 3296–3300
5. Stow, J. L., Kjellén, L., Unger, E., Höök, M. & Farquhar, M. G. (1985) J. Cell Biol. **100**, 975–980
6. Jalkanen, M., Nguyen, H., Rapraeger, A., Kurn, N. & Bernfield, M. (1985) J. Cell Biol. **101**, 976–984
7. Jalkanen, M., Rapraeger, A. & Bernfield, M. (1988) J. Cell Biol. **106**, 953–962
8. Laterra, J., Silbert, J. E. & Culp, L. A. (1983) J. Cell Biol. **96**, 112–123
9. Höök, M., Kjellén, L., Johansson, S. & Robinson, J. (1984) Annu. Rev. Biochem. **53**, 847–869
10. Woods, A., Couchman, J. R. & Höök, M. (1985) J. Biol. Chem. **260**, 10872–10879
11. Kanwar, Y. S., Linker, A. & Farquhar, M. G. (1980) J. Cell Biol. **86**, 688–693
12. Kobayashi, S., Oguri, K., Kobayashi, K. & Okayama, M. (1983) J. Biol. Chem. **258**, 12051–12057
13. Stow, J. L., Glasgow, E. F., Handley, C. J. & Hascall, V. C. (1983) Arch. Biochem. Biophys. **225**, 950–957
14. Kanwar, Y. S., Veis, A., Kimura, J. H. & Jakubowski, M. L. (1984) J. Cell Biol. **81**, 762–766
15. Klein, D. J., Brown, D. M. & Oegema, T. R., Jr. (1986) J. Biol. Chem. **261**, 16636–16652
16. Klein, D. J., Brown, D. M., Oegema, T. R., Brenchley, P. E., Anderson, J. C., Dickinson, M. A. J., Horigan, E. A. & Hassell, J. R. (1988) J. Cell Biol. **106**, 963–970
17. Kato, M., Koike, Y., Suzuki, S. & Kimata, K. (1988) J. Cell Biol. **106**, 2203–2210
18. van den Heuvel, L. P. W. J., Veerkamp, J. H., Monnens, L. A. H. & Schroder, C. H. (1988) Int. J. Biochem. **20**, 1391–1400
19. Kanwar, Y. S., Hascall, V. C. & Farquhar, M. G. (1981) J. Cell Biol. **90**, 527–532
20. Parthasarathy, N. & Spiro, R. G. (1984) J. Biol. Chem. **259**, 12749–12755
21. Shimomura, H. & Spiro, R. G. (1987) Diabetes **36**, 374–381
22. Hovingh, P., Piepkorn, M. & Linker, A. (1986) Biochem. J. **237**, 573–581
23. Templeton, D. M. & Khatchatourian, M. (1988) Biochem. Cell Biol. **66**, 1078–1085
24. Mason, R. M., Beavan, L. A. & Davies, M. (1988) in Progress in Basement Membrane Research: Renal and Related Aspects in Health and Disease (Gubler, M. C. & Sternberg, M., eds.), pp. 77–89, John Libbey Eurotext, Paris
25. Farquhar, M. G., Lemkin, M. C. & Stow, J. L. (1985) in Nephrology, vol. 1 (Robinson, R. R., ed.), pp. 580–600, Springer, New York
26. Hassell, J. R., Robey, P. G., Barrach, H., Wilczek, J., Rennard, S. I. & Martin, G. R. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 4494–4498
27. Martinez-Hernandez, A. & Amenta, P. S. (1983) Lab. Invest. **47**, 247–257
28. Timpl, R. & Dziadek, M. (1986) Int. Rev. Exp. Pathol. **29**, 1–112
29. Pejler, G. & David, G. (1987) Biochem. J. **248**, 68–77
30. Paulsson, M., Dziadek, M., Suchanek, C., Hunttner, W. & Timpl, R. (1985) Biochem. J. **231**, 571–579
31. Hassell, J. R., Leyshon, W. C., Ledbetter, S. R., Tyree, B., Suzuki, S., Kato, M., Kimata, K. & Kleinman, H. K. (1985) J. Biol. Chem. **260**, 8098–8105
32. Fujiwara, S., Wiedemann, H., Timp, R., Lustig, A. & Engel, J. (1984) Eur. J. Biochem. **143**, 145–157
33. Oohira, A., Wight, T. N., McPherson, J. & Bornstein, P. (1982) J. Cell Biol. **92**, 357–367
34. Reubsaet, F. A. G., Langeveld, J. P. M. & Veerkamp, J. H. (1985) Biochim. Biophys. Acta **838**, 144–150
35. Reubsaet, F. A. G., Veerkamp, J. H. & Monnens, L. A. H. (1985) Nephron **41**, 344–347
36. Langeveld, J. P. M. & Veerkamp, J. H. (1981) Comp. Biochem. Physiol. B **68**, 31–40
37. Farndale, R. W., Sayers, C. A. & Barrett, A. J. (1982) Connect. Tissue Res. **9**, 247–248
38. Parthasarathy, N. & Tanzer, M. L. (1987) Biochemistry **26**, 3149–3156
39. Wasteson A.. (1971) J. Chromatogr. **59**, 87–97
40. Lagunoff, D. & Warren, G. (1962) Arch. Biochem. Biophys. **99**, 396–400
41. Edge, A. S. B., Faltynek, C. R., Hof, L., Reichert, L. E. & Weber, P. (1981) Anal. Biochem. **118**, 131–137
42. Vaitukaitis, J. L. (1981) Methods Enzymol. **73**, 46–52
43. Louvard, D., Reggio, H. & Warren, G. (1982) J. Cell Biol. **92**, 92–107
44. Rennard, S. I., Berg, R., Martin, G. R., Foidart, J. M. & Gehron Robey, P. (1980) Anal. Biochem. **104**, 205–214
45. Faaber, P., Capel, P. J. A., Rijke, G. P. M., Vierwinden, G., Van de Putte, L. B. A. & Koene, R. A. P. (1984) Clin. Exp. Immunol. **55**, 502–508
46. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
47. Seno, N., Anno, K. & Kondo, K. (1970) Anal. Biochem. **37**, 197–202
48. Morrissey, J. H. (1981) Anal. Biochem. **17**, 307–326
49. Konat, G., Offner, H. & Mellan, J. (1984) Experientia **40**, 303–304
50. Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
51. Burnette, W. N. (1981) Anal. Biochem. **112**, 194–203
52. Hawkes, R., Niday, E. & Gordon, J. (1982) Anal. Biochem. **119**, 142–147

53. Aufderheide, E., Chiquet-Ehrismann, R. & Ekblom, P. (1987) J. Cell Biol. **105**, 599–608
54. Edge, A. S. B. & Spiro, R. G. (1987) J. Biol. Chem. **262**, 6893–6898
55. Lyon, M., Steward, W. P., Hampson, I. N. & Gallagher, J. T. (1987) Biochem. J. **242**, 493–498
56. Pietromonaco, S. F. & Farquhar, M. G. (1989) Kidney Int. **35**, 163
57. Spiro, R. G. & Mohan, P. S. (1987) in Renal Basement Membranes in Health and Disease (Price, R. G. & Hudson, B. G., eds.), pp. 11–23, Academic Press, London

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