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Heparan sulfate proteoglycan from human tubular basement membrane. Comparison with this component from the glomerular basement membrane

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Heparan sulfate proteoglycan (HSPG) was extracted from human tubular basement membrane (TBM) with guanidine and purified by ion-exchange chromatography and gel filtration. The glycoconjugate was sensitive to heparitinase and resistant to chondroitinase ABC, had an apparent molecular mass of 200–400 kDa and consisted of 70% protein and 30% glycosaminoglycan. The amino acid composition was characterized by its high content of glycine, proline, alanine and glutamic acid. Hydrolysis with trifluoromethanesulfonic acid yielded core proteins of 160 and 110 kDa. The heparan sulfate (HS) chains obtained after alkaline $NaBH_4$ treatment had a molecular mass of about 18 kDa. Results of heparitinase digestion and HNO_2 treatment suggest a clustering of sulfate groups in the distal portion of the HS side chains. These chemical data are comparable to those obtained previously on glomerular basement membrane (GBM) HSPG (Van den Heuvel et al. (1989) Biochem. J. 264, 457–465). Peptide patterns obtained after trypsin, clostripain or V8 protease digestion of TBM and GBM HSPG preparations showed a large similarity. Polyclonal antisera and a panel of monoclonal antibodies raised against both HSPG preparations and directed against the core protein showed complete cross-reactivity in ELISA and on Western blots. They stained all basement membranes in an intense linear fashion in indirect immunofluorescence studies on human kidneys. Based on these biochemical and immunological data we conclude that HSPGs from human GBM and TBM are identical, or at least very closely related, proteins.

Introduction

In recent years, substantial advances have been made in the knowledge of the important role of basement membranes in health and disease. In the kidney, the glomerular basement membrane (GBM) is a double membrane produced by both endothelial and epithlial cells, the tubular basement membrane (TBM) is synthesized by the tubular epithelial cells [1]. Extensive studies have defined the basic structure of basement membranes [2–5]. Type IV collagen [6,7] and heparan sulfate proteoglycans (HSPGs) [8–12] have been isolated from the GBM while the presence of laminin and entactin (nidogen) was demonstrated by immunohistochemical techniques [13]. The anionic sites formed by the HS side chains of HSPGs in the GBM maintain the permselectivity (charge- and size-selective barrier) and prevent clogging of the membrane by macromolecules [14–17]. Concerning HSPG in the TBM providing a strong and elastic mechanical support to the epithelium [18], little is yet known. Its concentration in the TBM is comparable to that of HSPG in GBM [19,20]. In polycystic kidney disease alterations in concentration and/or composition in TBM HSPG have been found [21,22]. In previous studies [23] we isolated and analyzed HSPGs from human glomeruli and tubules. Different HSPG populations however are present in glomeruli and GBM [24,25], and in tubules and TBM, respectively. Since we are primarily interested in the basement membrane variant, we purified and analyzed in the present study HSPG from human TBM and prepared specific mono-

Abbreviations used: EHS, Engelbreth-Holm-Swarm; GBM, glomerular basement membrane; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; TBM, tubular basement membrane; TFMS, trifluoromethanesulfonic acid.

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clonal and polyclonal antibodies against this proteoglycan. The present investigation was also aimed at obtaining information about a possible relationship of GBM and TBM HSPGs.

Materials and Methods

Materials

Sodium cyanoborohydride, pronase E and DC-Fertigplatte Kieselgel 60 were purchased from Merck, Darmstadt, F.R.G.; [^{14}C]formaldehyde (55.0 mCi/mmol) from Du Pont, New England Nuclear, Dreieichenhain, F.R.G.; clostripain and V8 protease from Sigma, St. Louis, MO, U.S.A.; trypsin from Boehringer, Mannheim, F.R.G. All other chemicals are described in detail elsewhere [12,23] or are of analytical grade.

Preparation of tubular basement membrane and isolation of proteoglycans

Human kidneys were obtained at autopsy within 20 h after death. Basement membranes were prepared from tubules by the detergent procedure previously described [23]. Proteoglycans were extracted from TBM and purified as described for proteoglycans from human GBM [12].

Radiolabeling of proteoglycan

Reductive alkylation with [^{14}C]formaldehyde (55.0 mCi/mmol) and sodium cyanoborohydride was carried out according to the procedure of Jentoft and Dearborn [26]. The reagents were removed by dialysis to yield a product with a specific activity of 25000 dpm/μg protein and 10850 dpm/μg protein for human TBM or GBM HSPG, respectively.

Radioiodination of proteoglycans was achieved with the chloramine T method [27]. The specific activity obtained was 20.9 μCi/μg for human TBM HSPG and 15.5 μCi/μg for human GBM HSPG.

Identification of proteoglycans

Proteoglycans were identified by treatment with heparitinase and chondroitinase ABC according to earlier described procedures [23].

TFMS and nitrous acid treatments of proteoglycan

Deglycosylation of native or ^{14}C-labeled proteoglycan (120000 dpm) was accomplished in 200 μl of TFMS-anisole (2 : 1, v/v) reagent under nitrogen for 3 h at 0 °C. Neutralization and extraction were carried out as previously reported [28] and followed by dialysis against distilled water.

Native or ^{14}C-labeled HSPG (120000 dpm) were treated with 200 μl of nitrous acid reagent [29] for 80 min at room temperature. The proteoglycan samples were dried and extracted three times with 80% ethanol.

Heparitinase and protease digestions

Native or [^{14}C]formaldehyde-labeled proteoglycans (180000 dpm) were incubated in 100 μl of 1 mM PMSF/10 mM *N*-ethylmaleimide/0.36 mM pepstatin/100 mM Tris-HCl/5 mM calcium acetate (pH 7.2) for 16 h at 37 °C with 10 mU heparitinase.

Digestion of ^{125}I- or [^{14}C]formaldehyde-labeled HSPGs with trypsin (50 μg/ml) was performed in 50 mM NH_4HCO_3 (pH 8.0), with V8 protease (20 μg/ml) in 20 mM Tris-HCl (pH 8.0) and with clostripain (2.5 U/ml) in 0.1 M Tris-HCl/0.1 M sodium acetate (pH 7.4). The digestions were carried out at 37 °C for 18 h.

Analysis of peptides

Two-dimensional separation of ^{125}I-labeled peptides on silica gel-coated thin-layer plates was carried out by the method of Elder et al. [30]. A sample in 5 μl of acetic acid/formic acid/water (3 : 1 : 16; by vol.) ($1 \cdot 10^6$ cpm) was resolved using electrophoresis in the first dimension and ascending chromatography in the second dimension. Electrophoresis was carried out at 950 V for 80 min in acetic acid/formic acid/H_2O (3 : 1 : 16; by vol.) using a flatbed apparatus (Pherograph original Frankfurt, type Mini 68, Vetter KG, Wiesloch, F.R.G.) at 4 °C. Chromatography was carried out in 1-butanol/pyridine/acetic acid/H_2O (13 : 10 : 2 : 8; by vol.) for about 5 h. Peptides were located by autoradiography with Kodak X-Omat R film.

Peptide mapping was also carried out with reverse-phase HPLC using a C_{18}-column (Lichrosorb 5RP18; 250 × 4 mm) and the following solutions, 0.1% trifluoroacetic acid and 0.1% triethylamine in water (solution A) and in acetonitrile (solution B). A linear gradient was used with a flow rate of 1 ml/min. The gradient was started at 0% B at 10 min, rose to 70% B at 150 min, and then to 100% B at 153 min. The solvent composition was held at 100% B for 30 min. For each chromatogram an amount of $2.0 \cdot 10^5$ dpm of ^{14}C-labeled digest of HSPG was injected. [^{14}C]Peptides were monitored by determination of radioactivity in the fractions.

Preparation and characterization of antibodies

Rabbit antisera against HSPG were prepared by a schedule of multiple intradermal injections of 50 μg [31]. Specificity and titer of the antisera were determined by ELISA [32,33]. The IgG fraction of the antisera was isolated with protein A-Sepharose CL-4B [34]. The inhibition ELISA was performed according to the procedure of Saku and Furthmayr [35].

For preparation of monoclonal antibodies BALB/c mice were injected intraperitoneally with 50 μg of TBM HSPG in 0.2 ml of a 1 : 1 mixture of complete Freund's adjuvant and phosphate-buffered saline. Four and 8 weeks after the first injection, each mouse received a rechallenge with the same amount of antigen in the

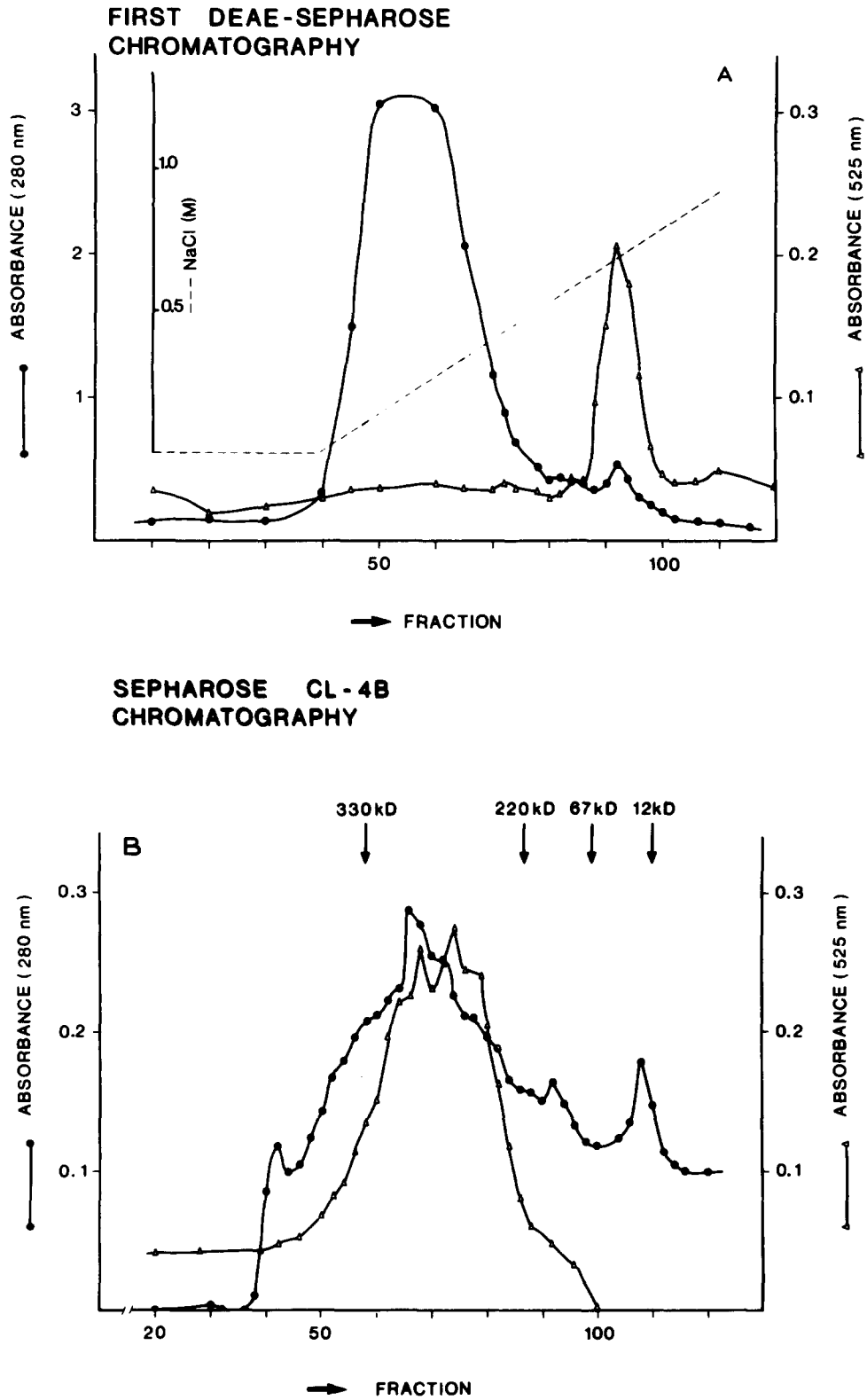

Fig. 1. (A) Chromatography of a human TBM extract on DEAE-Sepharose CL-6B (fast flow) Elution was performed with 7 M urea/10 mM Tris-HCl buffer (pH 6.8), followed by a gradient of 0–2 M NaCl (— — —) in the same buffer. Fractions of 8 ml were analyzed for protein (A_{280}, ●) and glycosaminoglycan (A_{525}, △). Fractions 86–104 were pooled for further purification. (B) Gel chromatography on Sepharose CL-4B of human TBM proteoglycan obtained after the second DEAE-Sepharose chromatography. A concentrated sample was applied to the column, which was eluted with 4 M guanidine-HCl/50 mM sodium acetate (pH 5.8). Fractions of 2.2 ml were collected and analyzed 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. Fractions 54–85 were pooled for further purification by Sephacryl S200 chromatography.

same mixture. After 10 weeks the mouse producing the highest antibody titer was injected with 20 μg HSPG intravenously daily during 3 days. On the subsequent day the spleen cells were fused with mouse myeloma cells using 40% polyethylene glycol [36]. Twelve days after fusion, the hypoxanthine-, aminopterin-, and thymidine- containing culture media from all wells were tested in ELISA and indirect immunofluorescence on 2 μm cryostat sections of a normal human kidney [37]. Hybrids producing specific antibodies were cloned by serial dilution [38], and re-examined for antibody production in ELISA and in indirect immunofluorescence studies.

Additional analytical procedures

Isolation of glycosaminoglycan chains and cellulose acetate electrophoresis were previously described [23]. Their molecular mass was determined by chromatography on a Sephadex G-100 column (1.6 × 72 cm) according to the procedure of Wasteson [39]. Polyacrylamide gel electrophoresis and immunoblot analyses of HSPG and core proteins were performed as described [12] Assays of protein, amino sugars, uronic acid and glycosaminoglycan content, as well as amino acid composition, were earlier described [23]. The sulfate content was measured by a modified benzidine method [40,41].

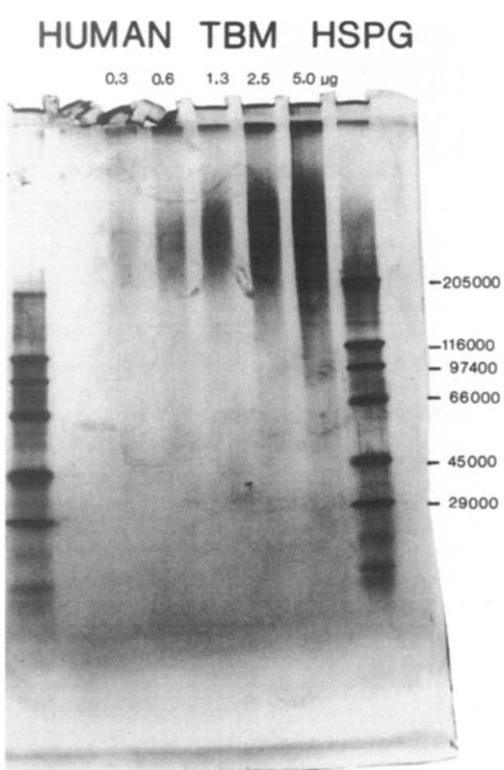

Fig. 2. SDS-polyacrylamide gel electrophoresis of human TBM HSPG in a 3–20% polyacrylamide slab gel. Per track 0.3, 0.6, 1.3, 2.5 or 5.0 μg of HSPG were applied and the gel was stained with silver. The left and right track contained molecular weight markers.

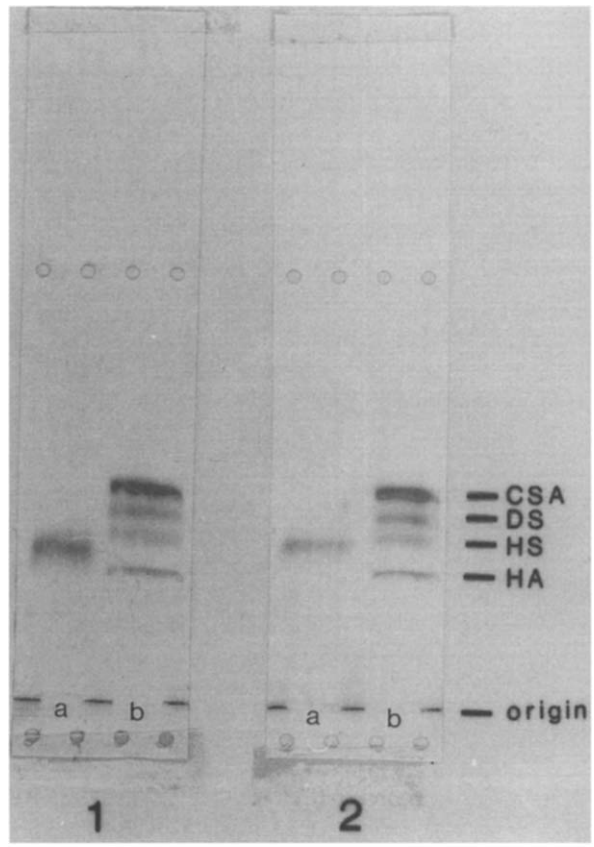

Fig. 3. Cellulose acetate electrophoresis in 0.1 M copper acetate buffer (pH 3.1) of the glycosaminoglycan fraction of HSPG from human GBM (lane 1a) and TBM (lane 2a). In lanes 1b and 2b a standard mixture containing hyaluronic acid (HA) from human umbilical cord, bovine kidney heparan sulfate (HS), porcine skin dermatan sulfate (DS) and whale cartilage chondroitin sulfate A (CSA) was applied.

N-Sulfated hexosamines were measured by the indole method [42] after nitrous acid treatment for 80 min as described above. The percentage of iduronic acid of the total uronic acid content was derived from the amount of unsaturated glycuronosyl residues formed after heparitinase digestion [43]. Galactose was determined with galactose dehydrogenase [44].

Results

Purification of HSPG from human TBM

Guanidine extraction of about 5 g TBM (originating from about 200 pairs of human kidneys) led to the solubilization of 62% of the glycosaminoglycan content of the TBM (2.42 mg/g dry weight; mean of two preparations). A comparable extraction efficiency was reported for HSPGs from rat and human GBM [8,12]. The solubilized proteoglycan was purified by DEAE-Sepharose chromatography (twice) and gel filtration on Sepharose CL-4B and Sephacryl S200 (Fig. 1A and B). The yield was approximately 1.5 mg proteoglycan/g TBM or 20% of the TBM HSPG content.

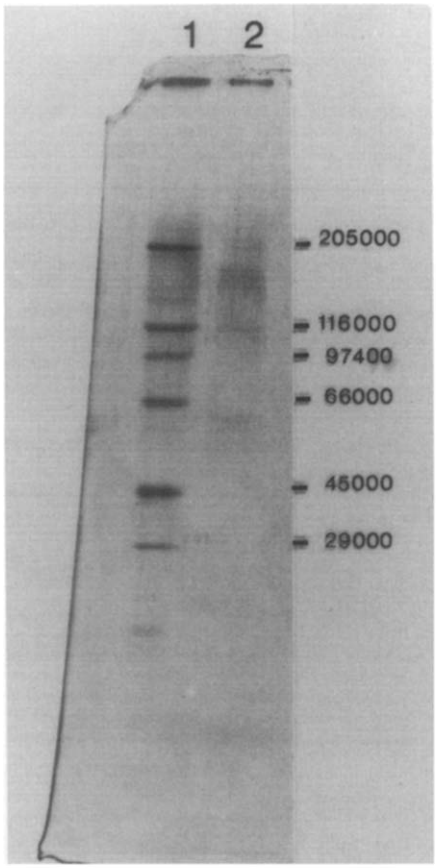

Fig. 4. SDS-polyacrylamide gel electrophoresis of the core protein (obtained after TFMS treatment) of human TBM HSPG in 3–20% polyacrylamide slab gel. Track 1 contained the molecular weight markers. In track 2, 5 μg of core protein was applied and the gel was stained with silver.

TABLE I

Amino acid composition of HSPG preparations from human TBM and GBM

Values are given as means (±S.D.) for the number of preparations between parentheses and are expressed as residues/1000 amino acid residues.

Amino acid	TBM (2)	GBM (5)
Aspartic acid (+asparagine)	84	86± 3
Threonine	54	54± 3
Serine	79	84± 4
Glutamic acid (+glutamine)	136	141± 7
Proline	81	84±13
Glycine	150	141±11
Alanine	89	99± 3
Valine	65	67± 2
Cysteine	5	5± 1
Methionine	6	7± 2
Isoleucine	26	22± 3
Leucine	72	68± 3
Tyrosine	24	20± 1
Phenylalanine	28	25± 0
Lysine	32	27± 2
Histidine	19	18± 2
Arginine	54	61±12

Characterization of TBM HSPG

The purity of the proteoglycans after Sephacryl S-200 was assessed by SDS-polyacrylamide gel electrophoresis, followed by Coomassie blue, silver staining or autoradiography of the [^{14}C]formaldehyde-labeled molecule. The intact proteoglycan migrated to a position between 200 and 400 kDa (Fig. 2). The gel did not contain additional bands indicating the absence of contamina-

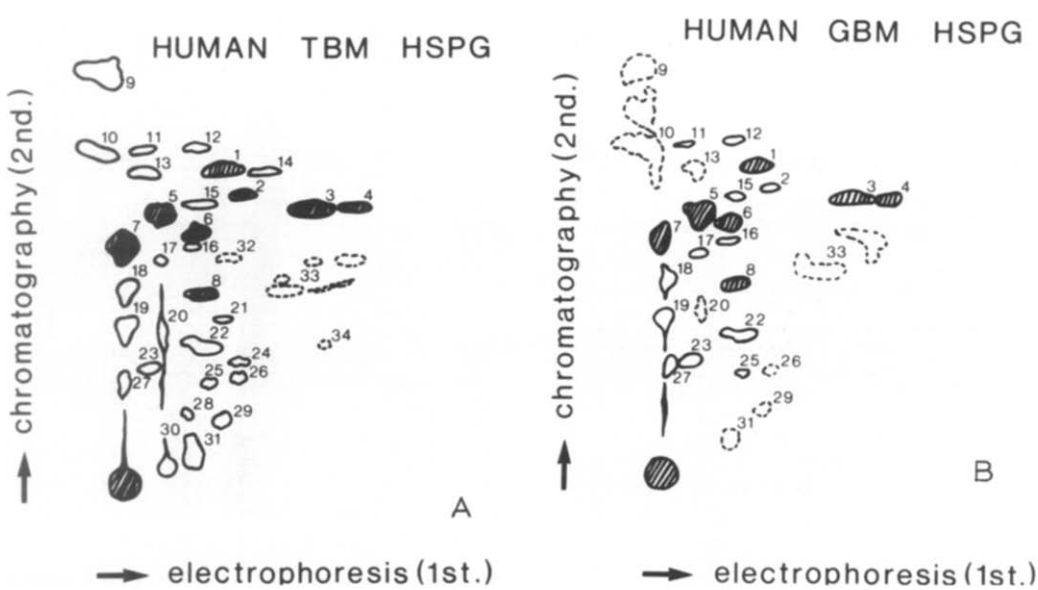

Fig. 5. A schematical representation of autoradiograms of the major ^{125}I-labeled peptides of trypsin-digested HSPGs from human TBM (A) or GBM (B). The intensity of the spots is indicated by their degree of darkness and lining.

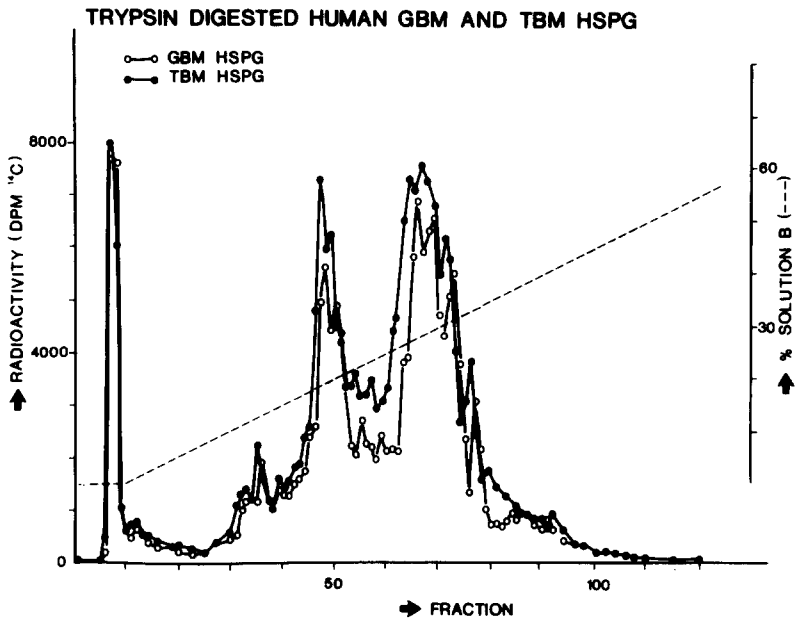

Fig. 6. Separation of the tryptic digest of [^{14}C]formaldehyde-labeled HSPGs from human TBM and GBM by HPLC on a C_{18} column.

tion with other proteins. The proteoglycan preparations did not react in ELISA with polyclonal antibodies against mouse laminin, which reacted well with human kidney in immunofluorescence. They also did not react with a polyclonal antibody against human type IV collagen or a monoclonal antibody against human laminin.

The electropherogram of the glycosaminoglycan samples prepared by alkaline $NaBH_4$ treatment of the proteoglycans from GBM and TBM showed one spot which migrated a little bit slower than the heparan sulfate standard from bovine kidney (Fig. 3). When the sample was treated with chondroitinase ABC or hyaluronidase prior to electrophoresis no effect was seen, but after treatment with heparitinase or nitrous acid, the spot was not longer discernible. Therefore the proteoglycans can be reliably identified as HSPGs. Chromatography of the HS chains on Bio-Gel P-30 or Sephadex G-100 indicated that their molecular mass is approximately 18 kDa (data not shown).

The core proteins of TBM HSPG obtained after TFMS treatment have molecular masses of 160 and 110 kDa (Fig. 4). Heparitinase- and nitrous acid treatments did not completely cleave the saccharide units from the core proteins and gave compounds with intermediate molecular masses (data not shown). These data indicate a clustering of *N*-sulfate groups in the distal part of the HS chains of the TBM HSPG as found for GBM HSPG [12].

TABLE II

Composition of HSPG preparations from human TBM and GBM

Values are given as means for two and five preparations from TBM and GBM, respectively. The range and S.D. value are also indicated, respectively.

Component	TBM		GBM	
	mg/100 mg	μmol/100 mg	mg/100 mg	μmol/100 mg
Protein	69 ±10	-	77 ±4	-
Glycosamino-glycan	31 ±10	-	23 ±4	-
Uronic acid	7.5± 0.6	39±4	9.2±2.0	47±11
Glucosamine	7.7± 0.3	45±4	8.3±1.7	46±10
Galactosamine	0.6± 0.5	4±3	0.5±0.6	3± 3
Galactose	2.1± 0.1	13±1	2.7±0.4	17± 3
Sulfate	4.6± 0.3	48±3	5.1±0.3	53± 7
N-Sulfate	1.7± 0.2	18±2	1.9±0.2	20± 2

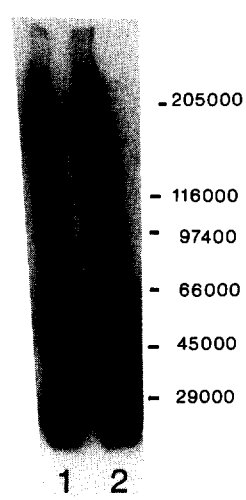

Fig. 7. Autoradiogram of a 3–20% SDS-polyacrylamide gel of the peptides obtained after V8-protease digestion of ^{125}I-labeled HSPG from human GBM (lane 1) and TBM (lane 2).

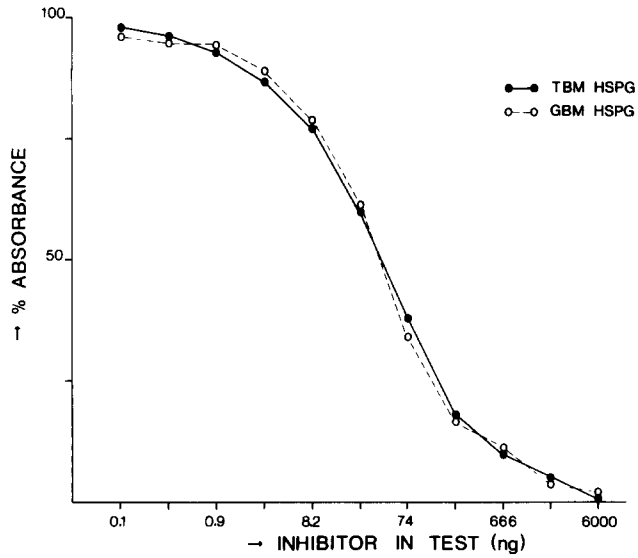

Fig. 8. Inhibition assay of TBM and GBM HSPG. ELISA plates were coated with TBM HSPG (0.5 μg/well). Anti-TBM-HSPG antibodies (10 μg/ml) were preincubated (16 h; 4°C) with various amounts (0.1–6000 ng) of TBM or GBM HSPG and subsequently tested for their binding to TBM HSPG.

Amino acid analysis showed, that the TBM HSPG has a composition similar to that of GBM HSPG (Table I). Amino acids typical for collagen, as hydroxyproline and hydroxylysine were absent in our HSPG preparations. Quantitative protein determination by amino acid analysis or by the Lowry procedure and glycosaminoglycan assay indicated that the proteoglycan molecule contains about 70% protein and 30% heparan sulfate. The sugar and sulfate composition of TBM HSPG was comparable to that of GBM HSPG and consistent with the occurrence of HS as the predominant saccharide chain (Table II). The presence of slight amounts of galactose suggests the occurrence of additional carbohydrate units. About 30% of the uronic acid residues were present in the iduronic acid form for TBM and GBM preparations.

Proteolytic digestion and peptide mapping

These procedures were applied to determine the structural relationship between the core proteins of human TBM and GBM HSPGs. The tryptic maps of both ^{125}I-labeled HSPG preparations (Fig. 5) show that almost all spots are identical for both preparations. In a second series of experiments we utilized HPLC to compare the peptide maps obtained after proteolytic digestions of ^{14}C-labeled TBM and GBM HSPGs. The results after trypsin digestion (Fig. 6) show again a similar pattern. Identical peptide maps were also observed for GBM and TBM HSPGs after V8 protease or clostripain digestion (data not shown). Finally we evaluated the peptide fragments generated by V8-protease digestion of ^{125}I-labeled TBM and GBM HSPGs by SDS-PAGE. The radioautogram (Fig. 7) shows again a clear similarity of the banding pattern of both preparations.

Immunological studies

Polyclonal antisera to intact TBM HSPG exhibited distinct titers of about 1 : 30 000 to 1 : 80 000 and recognized epitopes on the core proteins. The IgG fractions reacted similarly in ELISA and on Western blots, when tested with TBM or GBM HSPG. In order to determine more precisely the extent of relationship between both

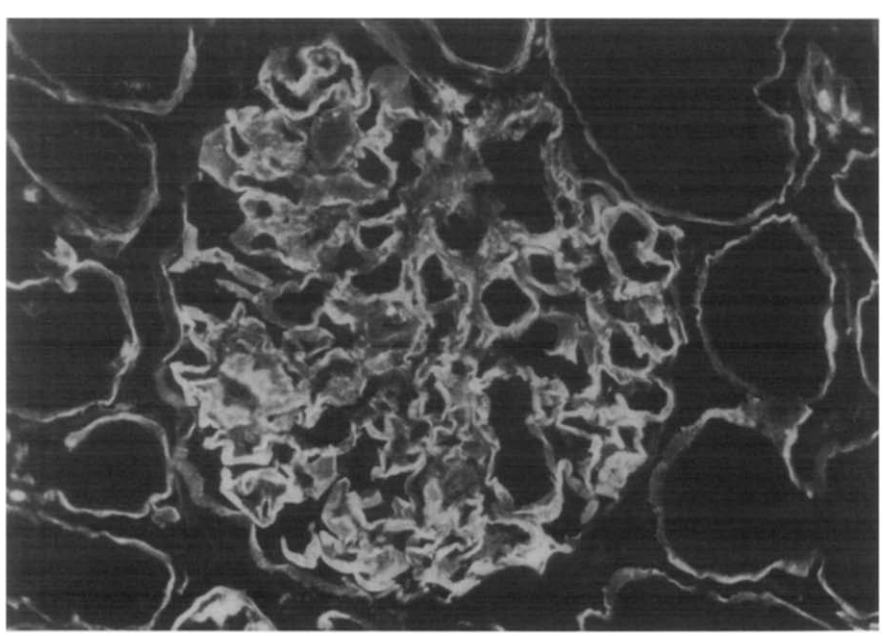

Fig. 9. Indirect immunofluorescence staining of human kidney tissue with a polyclonal anti-(human TBM HSPG) serum (dilution 1 : 200) (×80).

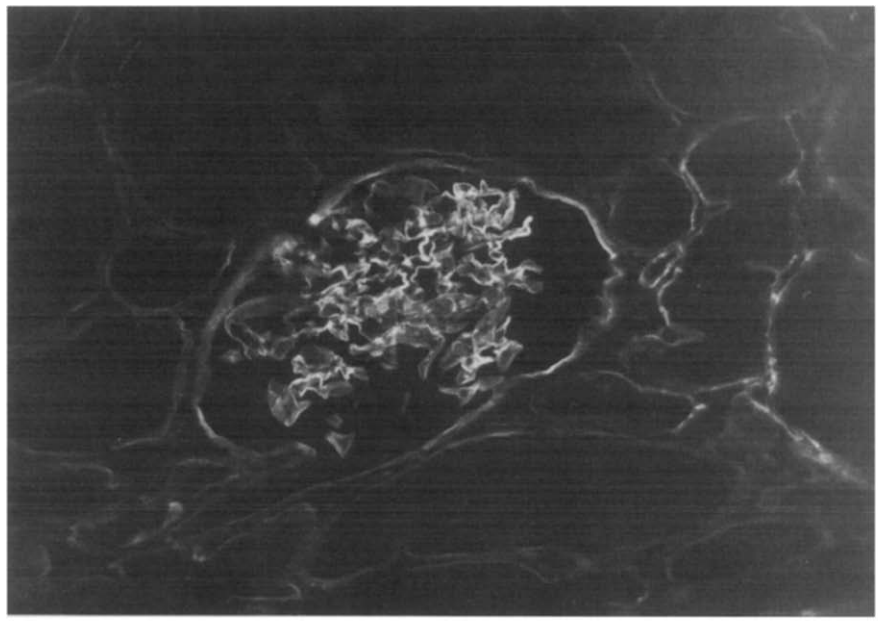

Fig. 10. Indirect immunofluorescence staining of human kidney tissue with a monoclonal anti-(human TBM HSPG) (×80).

antigens, we performed inhibition ELISA assays (Fig. 8). The similar slopes for the inhibition curves with 50% inhibition at 25–75 ng for both HSPG preparations indicate that the epitopes which are recognized by anti-TBM HSPG antibodies are shared by GBM HSPG. Similar results were obtained with the anti-GBM HSPG antisera and both HSPG preparations.

A panel of 25 mouse monoclonal antibodies against human TBM HSPG was selected for detailed study of epitope specificity. The antibodies showed no reactivity to the HS chains (obtained after pronase digestion), laminin, fibronectin or type IV collagen in ELISA. All monoclonal antibodies were directed against the core protein of TBM HSPG. They all showed complete cross-reactivity in ELISA and on Western blots with human GBM HSPG. Eighteen monoclonals against the latter compound cross-reacted also with TBM HSPG.

In indirect immunofluorescence studies the polyclonal antiserum against human TBM HSPG specifically stained all basement membranes of human kidney (Fig. 9) in an intense linear pattern, not only the TBM, but also GBM, peritubular capillary basement membranes and Bowman's capsule. The antibodies showed the same reaction pattern with human, horse, rat, mouse and hamster kidney (not shown). The reaction was the same as with the polyclonal antibodies against human GBM HSPG [12]. The monoclonal antibodies against human TBM HSPG also stained all basement membranes of the human kidney (Fig. 10), although the fluorescence in the GBM was more pronounced than that of the TBM. In contrast to the polyclonal antibodies the monoclonal antibodies were species specific and bound only to human kidney tissue.

Discussion

Chemical analyses of the uronic acid, amino sugar and sulfate content and degradation studies with HNO_2 and glycosidases indicated that heparan sulfate was the glycosaminoglycan chain of the proteoglycans of human TBM as for GBM [12]. On the base of the protein to glycosaminoglycan ratio (7 : 3) HSPG accounted for approximately 1% of the dry weight of TBM. The glycoconjugate had an apparent molecular mass of 200–400 kDa. Molecular mass estimates for basement membranes HSPGs vary widely from 750 kDa to 130 kDa for HSPG preparations from PYS-2 cells [45], HR9 cells [46], cultured endothelial cells [47,48], the murine EHS tumor [49], rat kidney glomeruli [50] or bovine GBM [51]. The range of the molecular mass of human TBM HSPG is in agreement with that reported for human GBM HSPG [11,12]. The molecular mass of 18 kDa for the HS chains from TBM HSPG is identical to that of HS chains from human GBM HSPG [12] and in the range of the molecular mass of these polymers from rat GBM (26 kDa) [8] and bovine GBM HSPG (14 kDa) [10]. However, the molecular mass of the HS chains of the HSPG from EHS tumor is clearly different (about 65 kDa) [52].

The presence of more core proteins after TFMS treatment of TBM HSPG may be due to proteolysis occurring during the extensive purification procedures or to proteolytic processing of a precursor protein. The same feature has been described for HSPGs from human GBM [11,12] and other origins [46,53]. Our data for the core proteins of human TBM HSPG are similar to those of GBM HSPG [11,12]. Bovine GBM HSPG

and rat GBM HSPG gave only one core protein of 128 kDa (after TFMS treatment) [51] and 130 kDa [54], respectively. Antibodies against EHS-tumor HSPG detected however in extracts of rat GBM, after treatment with heparitinase, core proteins of 250, 200 and 150 kDa [55].

The protein content of HSPG from human TBM is comparable with that from human and bovine GBM [10,12], but clearly different from that of rat GBM [8]. The amino acid and sugar composition of human GBM and TBM HSPG are quite similar and comparable to that of bovine GBM HSPG [10]. The amino acid composition appears also comparable to that of the low density HSPG from EHS tumor [56].

The peptide mapping data and the immunohistological and immunochemical results demonstrate a close structural relationship between human TBM and GBM HSPGs. Peptide mapping after proteolytic digestion (with 3 different specific proteases) and labeling with ^{125}I (which binds to tyrosine, histidine and phenylalanine [30]) and [^{14}C]formaldehyde (which binds to lysine [26]) resulted with three different separation techniques in just identical peptides. The results with the polyclonal and monoclonal antibodies on Western blots and in (inhibition) ELISA and indirect immunofluorescence studies did not reveal epitopes specific for one HSPG type. The presence of common antigenic determinants in the proteoglycans of GBM and TBM has been observed before. Polyclonal antibodies against the core protein of GBM HSPG from rat, man and cattle reacted also with TBM in indirect immunofluorescence studies [12,21,57]. Our data are consistent with these observations.

In conclusion, the biochemical and immunological data presented demonstrate a close structural and immunochemical similarity, especially with respect to the core protein, of the HSPGs from human GBM and TBM. These molecules may result from a partially processing of a precursor of the BM core protein. They could differentially be modified by glycosylation depending on the synthetic machinery of the cells [58]. Differences of saccharide chains would not be without functional consequences [59]. Resolution of the core protein characteristics (amino acid sequence, secondary structure, its functional domains and its mode of integration into the basement membrane) of basement membrane HSPGs will probably arise in the future by means of molecular biologic techniques. At present, polyclonal and monoclonal antibodies are important tools in probing the distribution and potential properties of these molecules.

Acknowledgements

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