Proteoglycan production by human glomerular visceral epithelial cells and mesangial cells *in vitro*

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Proteoglycans metabolically labelled with [³⁵S]sulphate and [³H]glucosamine or [³H]leucine were isolated from the incubation medium and cell layer of human adult mesangial cells and glomerular visceral epithelial cells using sequential DEAE chromatography purification steps followed by gel-filtration chromatography. The proteoglycan composition of each peak was analysed by treatment with HNO₃, chondroitinase ABC or chondroitinase AC followed by chromatography on Sephadex G-50 columns. Heparan sulphate proteoglycan (HSPG) and dermatan sulphate proteoglycan were detected in both the culture medium and cell layer of mesangial cells. Culture medium of glomerular visceral epithelial cells contained HSPG and a second proteoglycan with the properties of a hybrid molecule containing HS and chondroitin sulphate (CS). The cell layer contained HSPG and CSPG. Detailed analysis of the hybrid molecule revealed that it had an apparent molecular mass of 400 kDa. SDS/PAGE of hybrid molecules, after treatment with heparitinase and chondroitinase ABC, revealed a core protein of 80 kDa. Using 1.8 % polyacrylamide/0.6 % agarose-gel electrophoresis, we deduced that the HS and CS were independently attached to one core protein. Because glomerular-basement-membrane HSPG is thought to be derived from mesangial cells and glomerular visceral epithelial cells and this molecule is involved in several kidney diseases, we investigated its synthesis in more detail. Anti-(rat glomerular-basement-membrane HSPG) monoclonal antibodies (JM403) and anti-(human glomerular-

basement-membrane HSPG) polyclonal antibodies (both antibodies known to react with the large basement-membrane HSPG, perlecan) reacted strongly with HSPG obtained from both mesangial cells and glomerular visceral epithelial cells. However, the hybrid molecule did not react with these antibodies, suggesting that the HS side chain and the core protein were different from glomerular-basement-membrane HSPG. To quantify HS we performed an inhibition ELISA using mouse antibodies specific for glomerular-basement-membrane HS glycosaminoglycan side chains. Glomerular visceral epithelial cells produced significantly higher levels of HS (between 197.56 and 269.40 µg/72 h per 10⁶ cells) than mesangial cells (between 29.8 and 45.5 µg/72 h per 10⁶ cells) (three different cell lines; *n* = 3; *P* < 0.001). HS production by these cells was inhibited by cycloheximide, revealing that it was synthesized *de novo*. Expression of perlecan mRNA, demonstrated using reverse transcriptase PCR, was different in the two cell types. We conclude that glomerular visceral epithelial cells and mesangial cells have characteristic patterns of proteoglycan production. Glomerular visceral epithelial cells produced a hybrid proteoglycan containing CS and HS independently attached to its core protein. This molecule does not bind to monoclonal and polyclonal antibodies known to react with perlecan. This is the first quantification of an HSPG synthesized by both glomerular visceral epithelial cells and mesangial cells. Differences in mRNA levels between these cells were found.

INTRODUCTION

The renal glomerulus contains two extracellular matrices, the glomerular basement membrane (GBM) and the mesangial matrix [1], which are produced by the glomerular visceral epithelial cells, the glomerular endothelial cells and the mesangial cells. One important class of constituents of these matrices are the proteoglycans, of which chondroitin/dermatan sulphate proteoglycan CSPG/DSPG [2,3] and heparan sulphate proteoglycan (HSPG) [2,4,5] are the major molecules. The quantities and proportions of these constituents may vary between matrices produced by different cell types [6,7]. Proteoglycan molecules consist, in general, of a core protein and glycosaminoglycan side chains covalently attached to serine or asparagine residues on the core protein [8]. Alterations in the HSPG content of the

glomerulus are thought to play an important role in the pathogenesis of a variety of glomerular diseases associated with changes in GBM charge, proteinuria [9] and matrix accumulation. Several studies indicate that the HS glycosaminoglycan side chains contribute greatly to the negative charge observed on the GBM [10–12]. Kanwar et al. [2] showed leakage of ¹²⁵I-albumin into the urinary space after *in situ* heparitinase treatment of the basement membrane. We found that antibodies against GBM HS induce an acute selective proteinuria in rats [9]. This negative charge results from sulphate and carboxy groups distributed along the glycosaminoglycan side chains. There have been some reports that question the contribution of HS to glomerular charge selectivity [13–15]. Podocalyxin [16] and carboxy groups [17] could also play a significant role in this respect. However, a decreased GBM HS content was found to be accompanied by

Abbreviations used: HS, heparan sulphate; CS, chondroitin sulphate; DS, dermatan sulphate; HSPG, heparan sulphate proteoglycan; CSPG, chondroitin sulphate proteoglycan; DSPG, dermatan sulphate proteoglycan; GBM, glomerular basement membrane; DMEM, Dulbecco's modified Eagle's medium; δFCS, heat-inactivated fetal calf serum; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; RT-PCR, reverse transcriptase PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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increased permeability of the glomerular capillary walls, especially for anionic molecules such as albumin. It is thought that a change in negatively charged molecules in the GBM results in the albuminuria seen in many types of glomerulopathy including membranous glomerulonephritis [18], congenital nephrotic syndrome [19], systemic lupus erythematosus [20], minimal lesions and diabetic nephropathy [21–25]. Klein et al. studied the production of HS and CS/DS in both human fetal glomerular visceral epithelial cells [7] and human fetal mesangial cells [26,27] *in vitro*, by metabolic labelling with $\text{Na}_2^{35}\text{SO}_4$ and [^3H]glucosamine. Thomas et al. [28] published results of similar experiments using human adult mesangial cells, and these investigators observed differences between proteoglycans synthesized by fetal and adult cells. These differences mainly consisted of higher proportions of CSPG and HSPG produced by adult mesangial cells as compared with fetal mesangial cells. Also HSPGs synthesized by adult and fetal mesangial cells were not the same.

Therefore the first part of this paper is a detailed analysis of proteoglycan production by mesangial cells and glomerular visceral epithelial cells isolated from adult human kidneys. The two cell populations were investigated under the same conditions in order to exclude any experimental differences. These studies were performed using metabolic labelling of the cells with $\text{Na}_2^{35}\text{SO}_4$ and [^3H]glucosamine or [^3H]leucine followed by extensive purification steps.

The second part focuses on HSPG, because of the importance of this molecule in several kidney diseases. To our knowledge, no studies have shown quantitative differences in HS synthesis by glomerular visceral epithelial cells and mesangial cells. The earlier reports of HS quantification used metabolic labelling only. However, although this method is sensitive, it can give serious errors in proteoglycan levels if it is the sole method used. Recently, antibodies have been developed that specifically recognize the HS glycosaminoglycan side chains of GBM HSPG, and these antibodies can be used in a sensitive inhibition ELISA [25] to measure HS. Therefore, HS synthesis in both visceral epithelial cells and mesangial cells can be quantified *in vitro*, and peak fractions obtained by HPLC can be identified. Recent studies have demonstrated that these monoclonal antibodies have high reactivity against the large basement-membrane HSPG, perlecan. Our knowledge of the complete nucleotide sequence of perlecan [29] gives us the opportunity to investigate its expression in different tissues and cell types. Expression of perlecan mRNA by glomerular visceral epithelial cells and mesangial cells was investigated using reverse transcriptase PCR (RT-PCR).

MATERIALS AND METHODS

Cell culture

Mesangial cells and glomerular visceral epithelial cells were cultured using glomeruli obtained from human kidneys (donors aged between 1.5 and 66 years) which could not be used for transplantation because of anatomical abnormalities. Mechanical dissociation and sequential sieving were performed as described [30,31]. In brief, renal cortices were minced and passed sequentially through sieves of mesh 400 μm , 200 μm and 150 μm . The sieved material was then exposed to collagenase type-I A (Sigma Chemical Co., St. Louis, MO, U.S.A.) for 20 min at 37 °C. Collagenase was inactivated by the addition of cold Dulbecco's modified Eagle's medium (DMEM) (Seromed; Biochrom K.G., Berlin, Germany) containing 5% heat-inactivated fetal calf serum (δFCS) (Hyclone Laboratories, Logan, UT, U.S.A.). The sieved material was collected on a

80 μm sieve. This suspension of more than 90% decapsulated glomeruli was resuspended in DMEM containing 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 20% δFCS . Immediately after the first cellular outgrowth from the glomeruli, glomerular visceral epithelial cells appeared and were detached with PBS/EDTA (20 mM). These cells were subsequently grown in T75 flasks in DMEM containing 5% δFCS [31]. Outgrowing mesangial cells (generally appearing within 21 days of seeding) were selectively subcultured in 24-well plates as described [30]. The pure populations of cells were washed, treated with trypsin (0.05%) and cultured in T25 and T75 flasks (Greiner, Frickenhausen, Germany). Cells were used between the second and eighth passage. For labelling studies, cells were grown in either T75 flasks or 24-well plates. Culture media for ELISA were collected from cells grown in 12-well tissue culture plates (Costar, Cambridge, MA, U.S.A.).

Immunofluorescence studies

Both cell types were characterized morphologically by phase-contrast microscopy, and immunofluorescence was performed with different specific antibodies. Cells were grown on sterile glass coverslips in DMEM containing 10% δFCS . The cells were fixed in acetone for 5 min at 0 °C, air-dried and incubated with primary antibody for 30 min. After extensive washing with PBS, the cells were incubated with appropriate fluorescein isothiocyanate (FITC)-conjugated anti-IgG or IgM for 30 min in the dark. After three washes with PBS, the slides were mounted in 1,4-diazobicyclo[2.2.2]octane/glycerol, assessed for fluorescence at 340–380 nm and photographed through a Leitz microscope (Wetzlar, Germany).

The following antisera were used: FITC-phalloidin for actin (Sigma); mouse anti-cytokeratin monoclonal antibody (mAb) (RGE 53) and anti-desmin mAb (Eurodiagnostics, Apeldoorn, The Netherlands); rabbit anti-(human factor VIII) (von Willebrand factor) (Dakopatts); mouse anti-CD10 mAb (anti-CALLA) (Dakopatts); rabbit anti-(human CR1); anti-5D9 (TN9) specifically recognizing proximal tubular epithelial cells and TN10 specifically recognizing glomerular visceral epithelial cells [32] were a gift from Dr. G. Müller and Dr. M. Nesper (Medizinische Klinik, Tübingen, Germany); rabbit anti-(human collagen types I, III and IV) (Southern Biotechnology Associates, Birmingham, AL, U.S.A.); mouse anti-(human collagen type IV $\alpha 1$ and type IV $\alpha 3$) mAbs [33] were a gift from Dr. J. Wieslander (Statens Serum Institute, Copenhagen, Denmark); rabbit anti-(human fibronectin) (Sigma) and anti-laminin mAbs (Calbiochem, La Jolla, CA, U.S.A.); goat anti-(human HSPG) [25,34] and mouse anti-HS mAbs were produced as described previously [9,25].

Metabolic labelling

Confluent human mesangial cells and glomerular visceral epithelial cells were labelled in leucine- and sulphate-free DMEM supplemented with 2% δFCS . Metabolic labelling of cells in media supplemented with a dose response of δFCS revealed that a 2% concentration induced a maximal incorporation of the label (results not shown). *De novo* synthesis of proteoglycans was studied by dual labelling with $\text{Na}_2^{35}\text{SO}_4$ (100 $\mu\text{Ci}/\text{ml}$; carrier-free; NEN, Boston, MA, U.S.A.) and [^3H]glucosamine (25 $\mu\text{Ci}/\text{ml}$) or [^3H]leucine (50 $\mu\text{Ci}/\text{ml}$) (Amersham International, Amersham, Bucks., U.K.). After 24 h at 37 °C, the radioactive media were removed and the cells were washed three times with cold PBS. The incubation media and three washes were pooled and extensively dialysed against 0.5 M sodium acetate, pH 5.8, at 4 °C with the following protease-inhibitor

cocktail: 10 mM EDTA, 5 mM phenylmethanesulphonyl fluoride, 2 mM benzamidine, 50 mM β -aminohehexanoic acid, 0.5 unit/ml aprotinin and 5 mM iodoacetamide. The cells and extracellular matrix were scraped from the plastic dishes with a rubber 'policeman' and extracted for 24 h at 4 °C by gently shaking in 4 M guanidinium chloride containing 50 mM sodium acetate, pH 5.8, and the protease-inhibitor cocktail, after dialysis as above. Both cell-media and cell-layer fractions were finally dialysed against DEAE buffer (6 M urea, 50 mM Tris, 0.2% CHAPS and protease-inhibitor cocktail, pH 7.0). The first purification step involved a DEAE anion-exchange (5 ml). The fractions eluted in the salt gradient of 0.1–1.0 M NaCl in DEAE buffer were pooled, dialysed and loaded on to a DEAE anion-exchange Bio-Gel TSK DEAE-5PW HPLC column (75 mm $\times$ 7.5 mm; Bio-Rad, Richmond, CA, U.S.A.). After application of the sample, the column was extensively washed with DEAE buffer, and bound material was eluted in a linear NaCl gradient (0.1–1.0 M). A flow rate of 1 ml/min was used and 1.0 ml fractions were collected. ^{35}S and ^3H radioactivity were measured by adding aliquots of the fractions to fluorophore Ultima Gold (Packard) and counting in a Beckman LS-7500 scintillation counter. The conductivity of each fraction was measured using a CDM3 Radiometer conductivity meter (Copenhagen, Denmark) and expressed in milliSiemens (mS). Peak fractions were pooled, dialysed against distilled water at 4 °C, lyophilized and stored at -20 °C until use.

Degradation of proteoglycans

The ^{35}S -/ ^3H -labelled glycosaminoglycans were released from the proteoglycan protein core using 0.05 M NaOH in the presence of 1 M NaBH_4 for 1 h at 73 °C [35]. The reaction was stopped by dropwise addition of acetic acid to pH 7 followed by Sephadex G-50 column chromatography (bed size 0.9 cm $\times$ 25 cm), equilibrated with 0.5 M sodium acetate, pH 7.0, containing 0.2% CHAPS, 10 mg/ml BSA and 1 mg/ml heparin. The contents of the column void volume were used for the following analyses: glycosaminoglycan composition and reactivity in ELISAs for HS glycosaminoglycan and HSPG core protein.

Glycosaminoglycan composition

Glycosaminoglycans were analysed by treatment with low-pH (pH 1) HNO_3 [19,36,37] or chondroitinase ABC (2.5 munits) or AC (2.5 munits) (both from Sigma). ^{35}S -/ ^3H -labelled chains that were 10% less sensitive to chondroitinase AC than to chondroitinase ABC were designated DS, whereas those equally sensitive to both enzymes were referred to as CS. After treatment, the reaction mixtures were analysed using a Sephadex G-50 column (0.9 cm $\times$ 25 cm) as described above. Some proteoglycans were analysed in more detail using a Hiload 16/60 Superdex HR200 HPLC column (Pharmacia, Uppsala, Sweden) equilibrated with 0.5 M sodium acetate buffer, pH 7.0, containing 0.2% CHAPS. The column was calibrated with thyroglobulin (669 kDa), apoferritin (443 kDa), β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa) and carbonic anhydrase (29 kDa).

Electrophoresis in composite gels of 1.8% polyacrylamide/0.6% agarose was performed by the method of Carney et al. [38] with minor modifications. The gel was stained with 0.2% Toluidine Blue in 0.1 M acetic acid to visualize HSPG from bovine kidney, dried and exposed to X-ray film (Kodak) at -70 °C. The intensity of the bands on the autoradiogram was determined with a 2202 Ultrascan laser densitometer (LKB, Woerden, The Netherlands).

To determine the apparent molecular mass of some of the

proteoglycan cores, SDS/PAGE was used. The different proteoglycans were purified as described above and subjected to either chondroitinase ABC and/or heparitinase under the same conditions. The samples were boiled for 10 min and subjected to SDS/PAGE (5% gels) under reducing conditions.

ELISA of HS

HS was detected by an inhibition ELISA using mouse antibodies specific for HS glycosaminoglycan side chains as previously described by van den Born et al. [25]. In brief, a constant amount of mAb JM403 was diluted to a concentration giving 80% of the maximal ELISA signal and preincubated with an equal volume (60 μl) of HS (from bovine kidney; Seikagaku, Tokyo, Japan) at concentrations ranging from 0.2 to 100 $\mu\text{g/ml}$ for 1 h at 37 °C, followed by 1 h at 4 °C, and tested in the ELISA with HS as the coated antigen. ELISAs were performed over a linear range on the culture media of glomerular visceral epithelial cells and mesangial cells. For these studies cells were grown in 12-well tissue culture plates to confluence and incubated for 24 h in maintenance culture medium (DMEM containing 0.5% δFCS). They were then cultured for 3 days in DMEM containing 0.5% δFCS ; the used media were collected and cells counted using a Coulter counter (Coulter Electronics, Mijdrecht, The Netherlands). To study whether HS was synthesized *de novo*, replicate wells of cells were grown in 12-well tissue culture plates. The cells were washed three times with PBS and maintained for 24 h in DMEM containing 0.5% δFCS . Subsequently they were cultured in either maintenance medium alone or medium containing 1 $\mu\text{g/ml}$ cycloheximide. At timed intervals culture media were removed and HS was measured using the above ELISA. Cells were counted as above. Simultaneously, cells were grown with or without cycloheximide, washed and then cultured for additional periods in normal media, and HS in the media was determined.

Semiquantitative measurement of perlecan mRNA

Total RNA was isolated from glomerular visceral epithelial cells and mesangial cells grown in T25 flasks in medium as described by Chomczynski and Sacchi [39]. For PCR, oligonucleotide primers specific for human perlecan [40] corresponded to nucleotide positions 1234–1253 (P1, 5'-TTACATGCAGCCAA-ACTCGTC-3'; nested cDNA primer), 362–380 (P2, 5'-TTTC-GAGAGGTGTCCGAGGCT-3'; sense PCR primer) and 613–631 (P3, 5'-TTATCTTGGGAAGTGGGGCAC-3'; anti-sense PCR primer) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific [41] oligonucleotides corresponded to nucleotide positions 340–359 (G1, 5'-GAGATGATGACCTTT-TGGC-3'; nested cDNA primer), 4–22 (G2, 5'-GTGAAGGT-CGGAGTGTCAACG-3'; sense PCR primer) and 286–303 (G3, 5'-GGTGAAGACGCGAGTGGACTC-3'; antisense PCR primer). RT-PCR was performed with the help of the geneAmp thermostable RT-PCR kit (Perkin-Elmer Cetus). Reverse transcription of 1 μg of total RNA was performed at 70 °C for 15 min using a mixture of 10 ng each of primers P1 and G1 and 5 units of reverse transcriptase in 20 μl . Amplifications of divided cDNA samples representing approx. 0.4 μg of total RNA were carried out separately for 30 (GAPDH) or 35 (perlecan) cycles. The PCR mixture contained 100 ng of PCR primers (P2–P3; G2–G3) and all four dNTPs (200 μM each) in 100 μl of reverse transcriptase buffer containing 2.5 mM MgCl_2 . Temperature cycles were set as follows: 94 °C for 1 min, 50 °C for 1 min, 2 min for 72 °C. PCR products were resolved by agarose-gel electrophoresis.

Statistical analysis

Data for control and experimental groups are expressed as means $\pm$ S.D. Statistical analysis was performed using Student's *t* test for unpaired samples, and a *P* value of ≤ 0.05 was used to determine significance.

RESULTS

Characterization of human mesangial cells and glomerular visceral epithelial cells

Mesangial cells formed elongated cells in multilayers, whereas glomerular visceral epithelial cells formed a confluent monolayer of polygonal cells (Figures 1a and 1b respectively). The cells were incubated with specific antisera (Table 1) and examined by immunofluorescence microscopy. Distinctly different staining patterns were observed for the two types of cell. Glomerular visceral epithelial cells reacted positively with CD10 (anti-CALLA) [31] and mAbs specific for them, TN10 [32], but did not react with mAbs directed against the C3b receptor (CR1), proximal tubular epithelial cells (TN9) [32] or von Willebrand factor. Mesangial cells reacted negatively with CD10, cytokeratin and von Willebrand factor, but showed a uniform fluorescence of the actin filaments with FITC-phalloidin. Both types of cell gave positive extracellular-matrix staining for collagen type IV $\alpha 1$, fibronectin, laminin, HS and HSPG core protein when cells were cultured for 3 days (Table 1). Culturing mesangial cells for 3 days resulted primarily in cytoplasmic staining of HS in a filamentous pattern (Figure 1c). This staining could be abolished by addition of HS. Staining of HS on glomerular visceral epithelial cells cultured for 3 days was typically in the extracellular matrix (Figure 1d). Goat anti-mouse-FITC was used as a negative

Table 1 Marker antibodies used to define cultured glomerular visceral epithelial cells and mesangial cells and their staining by immunofluorescence

Antibody	Glomerular visceral epithelial cells	Mesangial cells
Anti-desmin	—	—
Anti-cytokeratin	+	—
Anti-CD10	+	—
Anti-CR1 (C3b receptor)	—	—
Anti-(von Willebrand factor)	—	—
Anti-SD9 (TN9)	—	—
TN10	+	—
Anti-phalloidin	Cytoplasm	Actin filaments
Anti-(collagen I)	—	+
Anti-(collagen III)	—	+
Anti-(collagen type IV $\alpha 1$)	+	+
Anti-(collagen type IV $\alpha 3$)	—	—
Anti-fibronectin	+	+
Anti-laminin	+	+
Anti-HS	+	+
Anti-(HSPG core protein)	+	+

control (Figure 1e). Staining of HSPG core protein gave similar patterns to those observed for HS. All mesangial cells but no glomerular visceral epithelial cells stained positively for collagen I and III (Table 1).

Incorporation of radioactively labelled sulphate and glucosamine into macromolecules

Glomerular visceral epithelial cells and mesangial cells were metabolically labelled for 24 h with $\text{Na}_2^{35}\text{SO}_4$ and $[^3\text{H}]$ glucosamine in sulphate-free medium supplemented with 2% δ FCS. After 24 h, the culture media were harvested. They contained 53.2–70.0% of the total incorporated radioactivity. The guanidinium chloride fraction containing both the intracellular components and extracellular matrix represented 30.0–46.7% of the total incorporated radioactivity. Less than 1% was left on the culture dishes after extraction. Both the cell supernatant and cell layer were purified twice by DEAE anion-exchange chromatography. The second purification step involved an HPLC column. For both cell types this resulted in the separation of two more or less distinct peaks (I and II) which were pooled as indicated in Figure 2. A portion of the total labelled macromolecules retained on the HPLC column were eluted at the beginning of the salt gradient (fractions 1–40). Glycosaminoglycans were not detected in these fractions.

Degradation of proteoglycans

The culture medium of mesangial cells contained almost equal amounts of HSPGs (peak I $50.8 \pm 2.8\%$ of the total proteoglycan production; $n = 3$) and DSPGs (peak II $49.2 \pm 2.8\%$ of total; $n = 3$) (Table 2A). The cell layer of mesangial cells also contained HS (peak I $58.2 \pm 4.7\%$ of total proteoglycan production; $n = 3$) and DS (peak II $41.8 \pm 5.7\%$ of the total; $n = 3$). Glomerular visceral epithelial cells synthesized different proteoglycans than did mesangial cells. The majority of the proteoglycan produced consisted of HSPG (peak I $61.5 \pm 5.1\%$ of the total). Peak II ($38.5 \pm 5.1\%$ of total) contained proteoglycans

Figure 1 Phase-contrast microscopy of glomerular cells in culture (a,b) and immunofluorescence microscopy of glomerular cells in culture stained with mAb JM403 (c,d)

(a,c) Mesangial cells; (b,d) glomerular visceral epithelial cells; (e) negative control (goat anti-mouse-FITC or rabbit anti-goat-FITC). Magnification for (a) and (b) $\times 100$; magnification for (c), (d) and (e) $\times 400$.

Figure 2 HPLC of ^{35}S -/ ^3H -labelled macromolecules synthesized by human mesangial cells (a,b) and glomerular visceral epithelial cells (c,d) in culture

Cells were labelled as described in the Materials and methods section. The culture medium and cell layer were harvested and extracted. The ^{35}S / ^3H -labelled macromolecules were first purified on a 5 ml DEAE anion-exchange column in DEAE buffer, pH 7.0. The second purification step involved an HPLC DEAE column. Profiles of the incorporated ^{35}S -labelled macromolecules from the culture medium (a,c) and the cell layer (b,d) are shown. Elution was with a linear NaCl gradient.

Table 2 Composition of proteoglycans produced by mesangial cells (A) and glomerular visceral epithelial cells (B) in culture

Numbers are percentage sensitivity; ELISA data are given as positive (inhibition > 30%) or negative (inhibition < 30%).

	Culture medium		Cell layer	
	I	II	I	II
A				
Chondroitinase ABC	0%	> 95%	0%	> 95%
Chondroitinase AC	0%	0%	0%	0%
HNO_2	> 95%	0%	> 95%	0%
Anti-HS	Positive	Negative	Positive	Negative
Anti-(HSPG core protein)	Positive	Negative	Positive	Negative
B				
Chondroitinase ABC	0%	35.4%	0%	> 95%
Chondroitinase AC	0%	34.3%	0%	> 95%
HNO_2	> 95%	50.1%	> 95%	0%
Anti-HS	Positive	Negative	Positive	Negative
Anti-(HSPG core portion)	Positive	Negative	Positive	Negative

sensitive to both chondroitinases and HNO_2 (Table 2B): 35.4% was sensitive to chondroitinase ABC, 34.3% to chondroitinase AC, and 50.1% to HNO_2 (Table 2B and Figure 3), revealing

either the presence of a mixture of CSPG and HSPG or a hybrid molecule with the different glycosaminoglycan side chains attached to one core protein. However, no positivity was observed in either the HS ELISA or the ELISA using polyclonal antibodies directed against the HSPG core protein, indicating that HS in this peak is different from that in peak I of the culture medium of these cells and is possibly not perlecan, as these antibodies have been shown to recognize this basement-membrane HSPG. The cell layer of these cells contained HS ($79.4 \pm 0.2\%$ of total; $n = 3$) and CS ($20.6 \pm 0.2\%$ of total; $n = 3$). All calculations were based on [^3H]glucosamine radioactivity.

Analysis of the CS- and HS-bearing proteoglycans

To investigate whether the contents of peak II of the culture medium of glomerular visceral epithelial cells contains a mixture of, or a hybrid molecule derived from, HSPG and CSPG, we metabolically labelled these cells with $50 \mu\text{Ci/ml}$ [^3H]leucine and $100 \mu\text{Ci/ml}$ $\text{Na}_2^{35}\text{SO}_4$ for 24 h in sulphate- and leucine-free DMEM containing 2% δFCS . The different proteoglycans were isolated as described in the Materials and methods section. Peak II (Figure 2c) was loaded on to a Superdex HR200 size-exclusion column equilibrated in 0.5 M sodium acetate buffer, pH 7.0, containing 0.2% CHAPS. A similar method was used to analyse a mixture of peak I and II from mesangial cells (Figure 2a), the latter serving as controls. Figure 4 shows the profiles. Peak II isolated from the culture medium of glomerular visceral epithelial

Figure 3 Sephadex G-50 gel-filtration chromatography of peak II of glomerular-visceral-epithelial-cell culture medium after purification by HPLC

Peak II was treated with NaBH_4 , subjected to Sephadex G-50 gel filtration (a), and the contents of the void volume subsequently analysed for reactivity with HNO_2 (b), chondroitinase ABC (c) or chondroitinase AC (d).

cells consists of one main peak and one small shoulder after Superdex HR200 separation (Figure 4a), whereas a mixture of peaks I and II isolated from mesangial-cell culture medium eluted as two peaks (Figure 4d). When the control mixture (peaks I and II of mesangial-cell culture medium) was treated with chondroitinase ABC (which cleaves both DS and CS chains), a small unsulphated [^3H]leucine peak appeared at K_{av} 0.142 (Figure 4e), which represents the CSPG core protein without the attached CS side chains. Treatment of the control mixture of mesangial-cell culture medium with HNO_2 resulted in another small unsulphated [^3H]leucine peak with a K_{av} of 0.127 (Figure 4f), representing the HSPG core protein. However, treatment of peak II from glomerular-visceral-epithelial-cell culture medium (HS/CS) with either HNO_2 or chondroitinase ABC produced no small unsulphated peaks. These findings suggest the presence of HS and CS in a hybrid molecule. Unfortunately no unsulphated peak was observed after treatment with both HNO_2 and chondroitinase ABC, but there was loss of radioactivity near the void volume. This peak is probably diluted in the large bed volume of the column.

SDS/PAGE was used to determine the size of the hybrid core protein (Figure 5). For this purpose peak II of glomerular visceral epithelial cells was treated with chondroitinase ABC,

heparitinase or both. After silver staining, several irrelevant protein bands appeared within the heparitinase control. The arrow identifies the sharp band observed between 97 and 66 kDa, which appeared after both enzyme treatments of peak II (Figure 5, lane 3). This band represents the core protein of the hybrid molecule. Note that it is specific, as it is not seen in lanes 5 and 6 in which the enzymes alone were analysed. No such 80 kDa band was generated when peak II was treated with either of the enzymes alone (lanes 2 and 4). As a control, peak I from glomerular-visceral-epithelial-cell culture medium was run both before and after heparitinase digestion. HSPG core protein migrated with a molecular mass of about 380 kDa (estimated by extrapolation; lane 7). To analyse whether the different types of glycosaminoglycan were independently attached to the core protein or attached via each other, 1.8% polyacrylamide/0.6% agarose-gel electrophoresis was performed. Large quantities of peaks I and II of the glomerular-visceral-epithelial-cell culture medium were purified (for purity see Figure 5) as described in the Materials and methods section. These peaks were labelled with ^{125}I in the presence of Iodobeads at room temperature for 20 min. The protein-bound iodine was separated from free iodine on a Sephadex G-50 column. When ^{125}I -labelled peak II was treated with heparitinase, there was a reduction (46.5%) (Figure

Figure 4 Analysis of peak II of glomerular-visceral-epithelial-cell culture medium

Peak II of glomerular-visceral-epithelial-cell culture medium was separated on a Hiload 16/60 Superdex HR200 column without treatment (a) or after treatment with chondroitinase ABC (b) or HNO_2 (c). Culture media from mesangial cells containing a mixture of HSPG and CSPG, not treated (d) or treated with chondroitinase ABC (e) or HNO_2 (f), were used as controls. —, ^3H ; ----, ^{35}S .

6, lane 4) in the intensity of the eluted band compared with the untreated material (Figure 6, lane 3). When peak II was treated with chondroitinase ABC a reduction in intensity (73.7%) of the original band was observed (lane 5). However, the band completely disappeared only when peak II was treated with both enzymes (lane 6). That the hybrid proteoglycan did not show a large change in migration in polyacrylamide/agarose after chondroitinase ABC treatment has been shown by Klein et al. [7]. It can be explained by the fact that only a few CS chains may have been present in the molecule (see also Figure 4; sensitivity to chondroitinase ABC was 35%). The different glycosamino-

glycan chains probably account for only a small proportion of the molecular mass of the intact proteoglycan (compare Figure 3a with Figure 3b). A relatively small CS chain may, however, account for a substantial proportion of the negative charge. This may explain the large decrease in intensity on the autoradiogram after chondroitinase treatment (Figure 6). This confirms that the different glycosaminoglycans (CS and HS) are independently attached to one core protein, as disappearance of the band would have occurred after only one of the enzyme treatments if the different glycosaminoglycans were attached to each other.

Figure 5 SDS/PAGE of DEAE-Sephacel- and Superdex HR200-purified HS and hybrid molecule synthesized by glomerular visceral epithelial cells

HSPG and the hybrid molecule bearing HS and CS were purified as described in the Materials and methods section. Equivalent quantities of the purified hybrid proteoglycans were subjected to SDS/PAGE (5% gel) under reducing conditions before (lane 1) and after digestion with heparitinase (lane 2), chondroitinase ABC (lane 4) or both (lane 3). The arrow indicates the position to which the core protein from the hybrid molecule without HS and CS migrated. HSPG isolated from the culture medium of glomerular visceral epithelial cells both before (lane 8) and after digestion with heparitinase (lane 7) was run as a control. Lanes 5 and 6 represent analysis of chondroitinase ABC and heparitinase respectively. Molecular-mass markers (kDa) are indicated on the right.

Figure 6 Gel electrophoresis of DEAE-Sephacel- and Superdex HR200-purified HS and hybrid molecule synthesized by glomerular visceral epithelial cells

HSPG and the hybrid molecule bearing HS and CS were purified as described in the Materials and methods section. Equivalent quantities of 125 I-labelled hybrid proteoglycans were subjected to electrophoresis on 1.8% polyacrylamide/0.6% agarose before (lane 3) and after digestion with heparitinase (lane 4), chondroitinase ABC (lane 5) or both (lane 6). As a control, 125 I-labelled HSPG isolated from glomerular visceral epithelial cells both before (lane 1) and after (lane 2) digestion with heparitinase were run. The arrow indicates the position to which HS from bovine kidney migrated.

Production of HS

Glomerular visceral epithelial cells and mesangial cells were cultured in 12-well plates to confluence in medium after which they were starved for 24 h in DMEM containing 0.5% δ FCS. The cells were then washed three times with PBS, and fresh medium was added to the wells. Three wells were treated with trypsin and the cells were counted in a Coulter counter to determine the number present at the beginning of the assay. After 3 and 6 days, the culture media (1 ml/well) were harvested and HS production was assessed. The results are means $\pm$ S.D. from three different cultures. Cell numbers did not change significantly during the experiment. Mesangial cells produced between 29.8

Table 3 Production of HS as quantified by ELISA

The cell line is denoted by the age of the donor.

Cell line	HS (μ g/72 h per 10^6 cells)
Mesangial	
48	36.8 $\pm$ 10.84
41	45.45 $\pm$ 6.17
66	29.78 $\pm$ 8.0
Glomerular visceral epithelial	
52	241.42 $\pm$ 54.83
25	269.40 $\pm$ 39.83
1.5	197.56 $\pm$ 17.86

Figure 7 Effect of cycloheximide (1 μ g/ml) on HS production by mesangial cells (a and b) and glomerular visceral epithelial cells (c and d) in culture

Cells were cultured for 3 days in the presence (+) or absence (—) of cycloheximide; culture media were then collected, and HS was determined by ELISA (a,c). Cells were washed, fresh culture medium was added and collected after 3 days and again tested for HS (b,d).

and 45.5 μ g/72 h per 10^6 cells (Table 3) when cultured for 3 days; glomerular visceral epithelial cells produced significantly higher levels (between 197.56 and 269.40 μ g/72 h per 10^6 cells ($P < 0.001$)). Evidence for *de novo* synthesis of HS was obtained by blocking the translation with cycloheximide. Cells were cultured for 72 h in the presence or absence of 1 μ g/ml cycloheximide and then culture media were collected and HS was assessed by ELISA. Cycloheximide inhibited HS production by mesangial cells from 39.1 ± 1.16 to 6.98 ± 0.68 μ g/72 h per 10^6 cells ($P < 0.001$) and by glomerular visceral epithelial cells from 241.42 ± 54.83 to 66.49 ± 17.89 μ g/72 h per 10^6 cells (Figure 7). The effects of cycloheximide were completely reversible 72 h after treatment, suggesting that the synthesis was *de novo* and that no cytotoxicity had occurred.

Expression of perlecan

Expression of perlecan in the two types of glomerular cell was analysed by the RT-PCR method. Cells were grown in T25 flasks in medium for 3 days, and total RNA was isolated. We used a gene-specific primer (nested cDNA primer) for cDNA synthesis which lies adjacent to, and downstream of, a pair of PCR primers used in the second step for PCR amplification. The

Figure 8 Agarose-gel electrophoresis of RT-PCR fragments

Fragments originating from GAPDH primers (lanes 4 and 5) or perlecan primers (lanes 2 and 3) of glomerular visceral epithelial cells (lanes 2 and 4) or mesangial cells (lanes 3 and 5) are shown. Bands were visualized by staining the 1.4% agarose gel with ethidium bromide. In lane 1 (λ HindIII) and lane 6 (100 bp ladder) molecular-mass markers were run.

specificity afforded by this methodology is evident from the presence of only one detectable PCR product of the expected size (269 bp). This method can be used for semiquantitative measurement of mRNA [41,42]. Both cell types show amplification of domain I of the human perlecan sequence (Figure 8). The GAPDH controls of the two cell types were more or less comparable. The intensity of the perlecan PCR fragment from mesangial cells is greater than that from glomerular visceral epithelial cells, suggesting higher steady-state perlecan mRNA levels in mesangial cells.

DISCUSSION

The two cell types used in this study were fully characterized on the basis of their morphology and immunofluorescence with different specific antibodies. The results confirm and extend earlier reports [1,30,31]. As the isolated glomerular preparation was decapsulated and virtually free of fragments of Bowman's capsule and tubules when studied by phase-contrast microscopy, a visceral rather than a parietal origin of the epithelial cells seems likely. Positive staining of the cultured glomerular visceral epithelial cells with TN10 confirms that these cells were visceral in origin [32].

In the present study we document that the major ^{35}S -/ ^3H -labelled macromolecules synthesized by adult mesangial cells and glomerular visceral epithelial cells are proteoglycans, but they are qualitatively and quantitatively different. Mesangial cells were found to produce both HSPG and DSPG, which were isolated from the culture medium and cell layer. Glomerular visceral epithelial cells produced HSPG and CSPG (almost equally sensitive to chondroitinase ABC and AC [7]). However, isolation of proteoglycans from the culture medium suggests the presence of a hybrid molecule bearing HS and CS (peak II, Figure 2c), as digestion of this peak demonstrated sensitivity to HNO_2 (50.1%), chondroitinase ABC (35.4%) and chondroitinase AC (34.3%) (Figure 3 and Table 2). For more detailed analysis, metabolic labelling with $\text{Na}_2^{35}\text{SO}_4$ and [^3H]leucine was performed. The isolated peak II was fractionated using molecular-sieve chromatography on a Hiloal 16/60 Superdex HR200 column. This resulted in a single peak (K_{av} 0.047). Treatment with either chondroitinase ABC or HNO_2 resulted in no large shift in a part of the ^3H -labelled peak, suggesting that only one core protein was present (Figure 4). A shift should have been observed as the expected molecular mass of the hybrid core protein is only 80 kDa [7]. The K_{av} of the fraction treated with

chondroitinase ABC or HNO_2 changed from 0.047 (400 kDa) to 0.053 (360 kDa) or 0.060 (330 kDa) respectively. When a mixture of HS and DS was fractionated on a Superdex HR200 column, a separate unsulphated [^3H]leucine peak was indeed observed (Figures 4e and 4f). If a mixture of CS and HS had been present in peak II, a separate unsulphated [^3H]leucine peak would have been observed as in Figures 4(e) and 4(f). Controls were also performed with a mixture of HSPG (isolated from the GBM of human kidneys [34]) and CSPG from whole shark cartilage (Sigma). The same effects of HNO_2 and chondroitinase ABC on this mixture using Superdex HR200 column fractionation was observed (results not shown). It was suggested previously [7] that glomerular visceral epithelial cells produce a hybrid molecule; however, this was not analysed in detail. This group also suggested that the CS/DS content of the hybrid molecule was smaller than the content of HS. This is confirmed by our analyses (50.1% sensitive to HNO_2 , 35.4% sensitive to chondroitinase ABC and 34.3% sensitive to chondroitinase AC). The hybrid molecule could be composed of HS and CS chains linked to one core protein or CS and HS attached via each other to one core protein. The fact that the sum of the HNO_2 and chondroitinase ABC sensitivities is less than 100% makes us favour the first possibility. To investigate this hypothesis, 1.8% polyacrylamide/0.6% agarose-gel electrophoresis was performed, and from the results it was deduced that HS and CS were independently attached to the core protein of the hybrid molecule. Rapraeger et al. [43] have previously reported another hybrid proteoglycan, syndecan-1, bearing both HS and CS produced by mouse mammary epithelial cells and Kato et al. [44] have also found a hybrid proteoglycan with the same core protein bearing HS and CS side chains. This latter group suggested that the CS content of a hybrid proteoglycan may be involved in modifying the potential binding activity of HS to other basement-membrane components. This feature may be important, as binding of HS to other basement-membrane components may be involved in several kidney diseases. The occurrence of a hybrid proteoglycan has also been described in more recent studies. Danielson et al. [45] isolated a hybrid proteoglycan synthesized by EHS-derived cells containing two to three HS chains and one CS chain attached to a large 400 kDa protein core. Isemura et al. [46] purified from human placenta a hybrid proteoglycan with a 800 kDa core protein and both HS and DS side chains. It is clear that the various hybrid proteoglycans described vary in molecular size and glycosaminoglycan content. Also differences in the size of the core protein were found. However, both the results of Klein et al. [7] and our studies suggest the production of a large hybrid proteoglycan containing both HS and CS side chains attached to a small 80 kDa core protein.

Up until now all studies on proteoglycan production by glomerular visceral epithelial cells and mesangial cells have been performed by metabolically labelling these cells with $\text{Na}_2^{35}\text{SO}_4$ and [^3H]glucosamine or [^3H]leucine. One of the advantages of this method is that the ratio between $\text{Na}_2^{35}\text{SO}_4$ and ^3H -labelled glycosaminoglycan or ^3H -labelled core protein can be studied in detail. Furthermore, this method allows the possibility of studying all types of proteoglycan produced by these cells, and determination of their molecular masses. However, quantification of metabolic labelling can lead to serious errors if it is the sole method used. As far as we know, it has not been possible to determine accurately the amount of HSPG produced. Using newly developed antibodies specific for GBM HS side chains in a sensitive inhibition ELISA, the production of HSPG by both glomerular visceral epithelial cells and mesangial cells can be measured more accurately. This will enable the study of factors that affect HSPG production. Results of such studies can then be

compared with those of $\text{Na}_2^{35}\text{SO}_4$ /[^3H]glucosamine or [^3H]leucine incorporation into macromolecules. This might allow differentiation between release of stored HSPG and *de novo* production and also comparison between the production of perlecan and non-perlecan HSPG. Recent studies performed in our laboratory show high reactivity of the mAbs used in this ELISA against the large basement-membrane HSPG (perlecan) (results not shown). RT-PCR demonstrates expression of perlecan in both cell types. As the method used theoretically cannot precisely reflect the steady-state level of perlecan mRNA, Northern-blot analysis was performed and revealed that glomerular visceral epithelial cells express mRNA at a lower level than mesangial cells (results not shown).

In summary, this study describes the production of DSPG and HSPG as well as expression of perlecan mRNA in mesangial cells. Glomerular visceral epithelial cells produce CSPG and HSPG and a hybrid molecule bearing HS and CS chains independently attached to its core protein. These cells also express perlecan. The majority of HSPG produced by the two cell types reacts with mAbs known to bind to perlecan, whereas the hybrid does not. Glomerular visceral epithelial cells produced significantly higher amounts of HS than do mesangial cells as revealed by the HS-specific ELISA.

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