Primary human glomerular endothelial cells produce proteoglycans, and puromycin affects their posttranslational modification

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Submitted 1 June 2004; accepted in final form 30 November 2004

The permselectivity of the glomerular filtration barrier is pivotal for the electrolyte and fluid balance in the body. The barrier has both charge- and size-selective properties (35, 36) but the question is where the different components of the barrier are located. Considerable focus has been directed to the glomerular basal membrane (GBM), which is built up by a three-dimensional network of mainly collagen, laminin, nidogen, and proteoglycans (PGs). The GBM has been said to be responsible for both size and charge selectivity (8, 24, 31, 48), but the charge-selective component has been questioned (4). Several novel proteins important for permselectivity have recently been discovered in the podocyte slit diaphragm (15, 43, 48). This has led to the podocyte now being the favorite candidate for constituting the filtration barrier. The endothelial cells of the glomerulus (GEC) are highly fenestrated and have thus not been considered to have prominent restrictive properties, apart from a few observations in the literature (41). However, endothelial cells in general are covered with a cell-surface coat containing PGs (10, 14, 28, 32, 52). PGs are constituted of a core-protein to which one or more polyanionic galactose- or glucosaminoglycan (GAG) chains are attached, the latter is heparan sulfate and the former is chondroitin sulfate. Plasma proteins tend to bind to the GAG chains and together they construct an extracellular matrix with both size- and charge-selective properties. In particular, the plasma protein orosomucoid seems to be vital for maintaining normal capillary permeability (7, 16) possibly via effects on the endothelial structure. It also has been demonstrated that orosomucoid is produced by endothelial cells per se (46). Moreover, using a novel fixation method, Røstgaard and Qvortrup (40) visualized an almost 300-nm-thick filamentous surface coating that appeared to be present over both fenestral and interfenestral surfaces of the glomerular endothelia. In addition, similar observations have been made in other organs (52).

A recent study from our group showed that enzymatic cleavage of hyaluronic acid and chondroitin sulfate resulted in a decreased amount of negatively charged fibers in the barrier and a significant increase of fractional clearance for albumin in mice. When studying the barrier by electron microscopy after the enzyme treatment, no damage to the glomerular cell structures was seen, which is compatible with the hypothesis that the endothelial cell-surface coat was degraded by the enzymes (22). Also, we showed that bovine glomerular endothelial cells produce negatively charged PGs containing heparan, chondroitin, and dermatan sulfate and that their synthesis is significantly decreased when exposed to puromycin amino nucleoside (PAN; a nephrosis-inducing agent) (45). We and others demonstrated that the synthesis of sulfate-containing GAGs by bovine GEC could be induced after stimulation with IL-1β (45) or transforming growth factor (TGF)-β (25). This upregulation could be a part of the cell defense by maintaining glomerular permselective properties (24) and may also be of significance in glomerular disorders characterized by matrix expansion. The information of the composition of the endothelial surface coat is still limited. Thus the production of PGs in the glomerular barrier has mainly been studied with metabolic sulfate labeling and enzyme degradation of the GAG chains. The method can reveal what class of GAG the cells produce but it is not possible to tell exactly what sort of PGs they express. It is of interest to investigate which kind of core proteins that are expressed in this system. Different classes of PGs are found extracellularly, transmembranely intercalated in cell membranes or via GPI anchors, and also intracellularly in storage granules (Table 1). In the present study, we wanted to...
explore the details of the PG core proteins produced by the human GECs. We were also interested in the effects of PAN on the PG core proteins and on key transferases involved in the synthesis of PGs, galactose and GAGs.

MATERIALS AND METHODS

Medium and Reagents

Glomerular endothelial cells were cultured in culture flasks (Costar, Cambridge, MA) in a humidified, 5% CO2 atmosphere at 37°C. The culture flasks were coated with attachment-factor (gelatin) from Cascade Biologics (Portland, OR). The medium used was RPMI 1640 culture flasks were coated with attachment-factor (gelatin) from Cascade Biologics (Portland, OR). The medium used was RPMI 1640 with supplements as follows: penicillin, streptomycin, and amphoter-cin B (PSA solution, 1 ml) from Cascade Biologics, 10% human serum, bovine brain extract (BBE), 700 μg/ml heparin (Cascade Biologics, Portland, OR). The remaining cells (2 flasks per group) were used to extract the glomeruli. Stainless steel sieves (Endecotts, London, UK) in the size of 180, 106, and 75 μm were used to extract the glomeruli.

Isolation of Glomeruli

Studies were performed on adult human kidney tissue removed by nephrectomy (due to stenosis or tumors). The study was approved by the local ethical committee at Göteborg University (R110 –98). Glomeruli were isolated from healthy tissue under sterile conditions by a gradual sieving procedure done according to the protocol by Balle-mann (2). The collected glomeruli were treated with 1 mg/ml collagenase type IV and V (Sigma, St. Louis, MO) were used to digest the whole glomeruli. Collagenase type IV and V (Sigma, St. Louis, MO) were used to digest the whole glomeruli. Stainless steel sieves (Endecotts, London, UK) in the size of 180, 106, and 75 μm were used to extract the glomeruli.

Characterization

Morphology. Outgrowths from collagenase-treated glomerular remnants and subcultures were examined with a phase-contrast microscope.

Acetylated low-density lipoprotein uptake. The cells were seeded onto chamber-slides (Falcon, Becton Dickinson, Meylan Cedex, France) and allowed to grow under normal conditions until they reached 50% confluence. The medium was removed and Dil-Ac-LDL (10 μg/ml; Biomedical Technologies, Stoughton, MA) was added to each well. After incubation for 3 h at 37°C, the cells were washed with 0.01 M PBS, pH 7.4. After fixation with 4% paraformaldehyde for 15 min, the cells were rinsed, mounted, and examined by fluorescence microscopy at 570 nm.

Ulex europaeus agglutinin I staining. The cells were seeded onto chamber-slides. When they reached 30–50% confluence, they were fixed with 4% paraformaldehyde for 15 min at room temperature (RT). After being rinsed with 0.01 M PBS, the cells were incubated with 2% BSA for 30 min at RT. Rhodamine Ulex europaeus agglu-
tinin I (Vector Laboratories) diluted 1:200 in the blocking solution was applied and the cells were incubated for 30 min at RT. The cells were rinsed, mounted, and examined by fluorescence microscopy at 570 nm. Human dermal fibroblasts were used as negative control cells.

Expression of endothelin-1. The RNA from the cells was analyzed by PCR for the specific sequence for endothelin-1. Sense primer was 5′-TGGACATCATTTGGGCAACA-3′ and antisense primer was 5′-TCTCTTGAGCTATGCTCC-3′.

Cell Culture and Stimulation with PAN

Human glomerular endothelial cells (passage 4) originally derived from the healthy poles of two kidneys (one male patient born -53 and one female born -48) were seeded into eight 25 cm2 flasks or two six-well plates and allowed to grow to 70% confluence. Cells in four flasks and one six-well plate of nonstimulated cells grown under normal conditions (i.e., “starved” in standard media with 2% human serum and half the amount of BBE). Stimulation was performed for 48 h at 37°C in a humidified, 5% CO2 atmosphere. After the experiment, cells from two flasks and the six wells for each group were trypsinized and used to prepare total RNA according to the RNeasy Mini protocol (Qiagen, VWR, Stockholm, Sweden). The remaining cells (2 flasks per group) were washed with PBS and then lysed by a buffer containing 1% SDS, Complete mini protease inhibitor (Roche), and 10 mM Tris, pH 7.4.

Real-Time PCR

Reverse transcription of 1 µg of RNA from both PAN and control groups was performed in avian myeloblastosis virus reverse transcriptase (AMV RT) buffer with AMV RT, dNTP (deoxy-CTP, -GTP, -TTP, and -ATP), random hexamers, and RNase inhibitor to a final volume of 80 µl (all reagents from Roche). The reverse transcription reaction was carried out at 25°C for 5 min, 42°C for 50 min, and 70°C for 5 min. The mRNA level of each PG was quantified by real-time PCR on the ABI Prism 7700 Sequence Detection system (TaqMan, Applied Biosystems ABI, Foster City, CA). Briefly, this assay uses the 5′-nuclease activity of Taq polymerase to cleave a reporter dye from a nonextendable hybridization probe during the extension phase of the PCR reaction. This ends the activity of a quencher dye and the reporter dye starts to emit fluorescence which increases in each cycle proportional to the rate of probe cleavage and is monitored in real-time. The threshold cycle (Ct) is defined as the fractional cycle number at which the reporter fluorescence reaches a certain level (i.e., usually 10 times the standard deviation of the baseline). Oligonucleotide primers and TaqMan probes were designed by using Primer Express version 1.5 (ABI), based on the GenBank database for syndecan, glypicain, versican, decorin, biglycan, and perlecan (Table 2). Primers and probes for the enzymes for chondroitin sulfate biosynthesis: chondroitin 4-O-sulfotransferase (ST), accession no. NM_015913, chondroitin 6-O-ST (NM_004273), chondroitin syn-

Table 1. Classes of PGs expressed by the human glomerular endothelial cells

<table>
<thead>
<tr>
<th>Proteoglycan</th>
<th>SYND (1–4)</th>
<th>GLYP (1–5)</th>
<th>VERS (0–3)</th>
<th>DECOR</th>
<th>BIGL</th>
<th>PERL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Core protein, kDa</td>
<td>22–45</td>
<td>~60</td>
<td>72–370</td>
<td>~40</td>
<td>~40</td>
<td>400–467</td>
</tr>
<tr>
<td>Number of chains</td>
<td>1–3 CS 1–2 HS</td>
<td>1–3 HS</td>
<td>0–23 CS</td>
<td>1 CS/DS</td>
<td>1–2 CS/DS</td>
<td>1–3 HS</td>
</tr>
<tr>
<td>Gene family name</td>
<td>Cell-associated PGs</td>
<td>Cell-associated PGs</td>
<td>Hyaluronan</td>
<td>SLRP (Small</td>
<td>Secreted</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>Destination</td>
<td>Membrane bound</td>
<td>Membrane bound</td>
<td>and lectin binding PGs</td>
<td>Leucine-rich PGs</td>
<td>Secreted</td>
<td>PGs</td>
</tr>
<tr>
<td>TaqMan gene expression normalized to β-actin*</td>
<td>1.59</td>
<td>1.33</td>
<td>1.43</td>
<td>0.87</td>
<td>0.78</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*The amount of target genes and endogenous control was determined from the corresponding standard curves. See MATERIALS AND METHODS. PGs, proteoglycans.

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Table 2. Oligonucleotide primers and probes used for real-time quantitative PCR

<table>
<thead>
<tr>
<th>Gene (Accession No.)</th>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Start</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syndecan-1 (NM_002997)</td>
<td>Forward</td>
<td>GCC GCA AAT TGT GCC TAC TAA</td>
<td>315</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGC CUG AGA AGT TGT CAG AGT C</td>
<td>391</td>
</tr>
<tr>
<td></td>
<td>Probes</td>
<td>(FAM)CCCTGCCCTTTGAGCAGAGAGGC-(TAMRA)</td>
<td>337</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>TGG CTG ACA GCA TCT CAG GA</td>
<td>209</td>
</tr>
<tr>
<td>Glypican-1 (NM_002081)</td>
<td>Forward</td>
<td>TGC CTT GAC TAT TGC GAG CA</td>
<td>1,023</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAT GGA GTC GAG GAT TCT CT</td>
<td>1,112</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>(FAM) GAGTGTGTTCCATCTGACCC- (TAMRA)</td>
<td>1,068</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>AAG GGC ACT ATG AGT CTC CTT CT</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>AAA GTC AAA TAA GGA TCT GGA TTA</td>
<td>137</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>(FAM) TTTGCAAGTTTCTGGCCTGACG- (TAMRA)</td>
<td>85</td>
</tr>
<tr>
<td>Versican (v0–v3) (NM_004385)</td>
<td>Forward</td>
<td>AGC ATG TGT ATT GTT ATG TGG ATC ATC T</td>
<td>982</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGC CTC GTC GAA GGT GAA TTA A</td>
<td>1,064</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>(FAM) TGATGTTGGCCATCTGACCC- (TAMRA)</td>
<td>1,016</td>
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<tr>
<td></td>
<td>Probe</td>
<td>AAA GTC AAA TAA GGA TCT GGA TTA</td>
<td>137</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>(FAM) TTTGCAAGTTTCTGGCCTGACG- (TAMRA)</td>
<td>85</td>
</tr>
<tr>
<td>Decorin (XM_045925)</td>
<td>Forward</td>
<td>GTC TCT GCT GCC GGT CAG C</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCA TGA ATG GCC CAT CTT G</td>
<td>226</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>(FAM) GCCGGCTTTGAGAGAGC- (TAMRA)</td>
<td>167</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>TGG CTG ACA GCA TCT CAG GA</td>
<td>209</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>GCA TGG AGC GAG TGA ATT TCA</td>
<td>323</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>(FAM) ACTTCCAGATGGTTTATTTCCGAGCCCTG-(TAMRA)</td>
<td>273</td>
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Table 3. Amplification efficiency for TaqMan primer/probe sets

<table>
<thead>
<tr>
<th>Gene</th>
<th>Slope</th>
<th>Intercept</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>3.329</td>
<td>24.1</td>
<td>0.992</td>
</tr>
<tr>
<td>Perlecan</td>
<td>3.608</td>
<td>27.56</td>
<td>0.995</td>
</tr>
<tr>
<td>Glypican</td>
<td>3.395</td>
<td>31.87</td>
<td>0.99</td>
</tr>
<tr>
<td>Versican</td>
<td>3.178</td>
<td>25.43</td>
<td>0.992</td>
</tr>
<tr>
<td>Syndecan</td>
<td>3.404</td>
<td>30.53</td>
<td>0.995</td>
</tr>
<tr>
<td>Biglycan</td>
<td>3.847</td>
<td>37.87</td>
<td>0.983</td>
</tr>
<tr>
<td>Decorin</td>
<td>3.405</td>
<td>32.29</td>
<td>0.985</td>
</tr>
<tr>
<td>CS-synthase 1</td>
<td>3.041</td>
<td>27.29</td>
<td>0.998</td>
</tr>
<tr>
<td>CS-4-OST</td>
<td>3.77</td>
<td>33.19</td>
<td>0.998</td>
</tr>
<tr>
<td>CS-6-OST</td>
<td>3.344</td>
<td>31.26</td>
<td>0.991</td>
</tr>
<tr>
<td>EXT-2</td>
<td>3.474</td>
<td>28.84</td>
<td>0.991</td>
</tr>
<tr>
<td>NDST</td>
<td>3.722</td>
<td>28.71</td>
<td>0.999</td>
</tr>
<tr>
<td>HST-2</td>
<td>4.066</td>
<td>32.24</td>
<td>0.997</td>
</tr>
<tr>
<td>HS-3OST1</td>
<td>3.514</td>
<td>29.21</td>
<td>0.998</td>
</tr>
<tr>
<td>HST-6</td>
<td>3.271</td>
<td>37.85</td>
<td>0.97</td>
</tr>
</tbody>
</table>

The table illustrates the standard curve equation for each gene where concentration is plotted on the x-axis and Ct on the y-axis. R² is calculated from the standard curve of 8 data pairs (0–200 ng).

Western Blot Analysis

The protein concentration was determined by using the BCA-Protein Assay Reagent kit (Pierce, Rockford, IL). Half of the amounts from the protein samples were digested with enzymes: chondroitinase ABC (0.1 U/sample, Sigma), heparitinase I (10 U/sample, Sigma) and heparitinase III (1 U/sample, Sigma) in ABC-buffer (100 mM Tris-HAc, 10 mM EDTA, 3 mM CaCl₂, pH 7.3) at 37°C for 7 h. Samples (~10 µg of each) were separated on Novex (San Diego, CA) precast 4–12% Bis-Tris gels for syndecan or 5% Tris-HCl gels (Bio-Rad, Hercules, CA) for versican and perlecan. After separation, the proteins were transferred to either polyvinylidene difluoride membrane (syndecan) or nitrocellulose membranes (versican and perlecan). Membranes were blocked with 5% nonfat dry milk in TBS-T (30 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween 20). The following primary antibodies, dilutions, and incubation times were used: rabbit anti-syndecan-1 (2 µg/ml, 1 h at RT; Zymed, San Francisco, CA), mouse anti-versican (2 µg/ml, 2 h at RT; Zymed) and rabbit anti-versican [1:2,000, 2 h at RT; a kind gift from Dr. D. Zimmermann, Dept. of Pathology, Zurich, Switzerland (54)]. After being rinsed, membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham Life Science, Amersham, UK). Immunoreactive bands were visualized using ECL+ (Amersham) and a CCD camera (LAS1,000; Fujifilm, Tokyo, Japan). The intensity of bands was measured by densitometry (Image Gauge V.3.45) and compared between the groups.

Biolabeling of GAG

Sulfate labeling. Human glomerular endothelial cells (passage 5) were allowed to grow to 70% confluence in 24-well plates before the media was changed. Cells in four wells were stimulated with PAN (1 µM) and as control we used four wells of nonstimulated cells grown under normal conditions (i.e., “starved” in standard media with 2% human serum and half the amount of BBE). Stimulation was performed for 48 h at 37°C in a humidified, 5% CO₂ atmosphere. Metabolic labeling of GAGs were obtained by adding 10 µCi/ml of [35S]sulfate labeled with this procedure.

Colabeling with sulfate and tritiated glucosamine. Human glomerular endothelial cells (passage 8) were allowed to grow to 70%
confluence in 24-well plates before the media was changed. The cells were starved and stimulated as described above. Metabolic colabeling with α-[6-3H]glucosamine (20 μCi/ml) and sulfate-35 (30 μCi/ml) (Amersham Biosciences, Uppsala, Sweden) was made to measure both the sulfate content and the length of the GAG chains.

A time course study was previously done to determine the metabolic activity of glomerular endothelial cells (45). After the stimulation, conditioned media were collected for further analysis.

**Analysis of PGs Synthesized and Released to the Media**

**Ion exchange and molecular sieve chromatography.** SULFATE LABELLED MOLECULES. PGs in the media were separated on the basis of differences in charge density, by using an ion-exchange chromatography di-ethyl-amino-ethyl (DEAE)-Sephacel column (0.7 × 2.5 cm, Amersham Bioscience). Cold carrier (4-O-S-Chondroitin sulfate, 100 μg, Seikagaku, Tokyo, Japan) was added to each sample to decrease the loss of labeled PGs in the separation process (34). After application of media containing [35S]sulfate-labeled PGs from control and PAN-treated cells, unbound radioactivity was removed from the column by washing with urea buffer, and PGs were eluted with 4 M guanidine hydrochloride, 50 mM NaOAc, 0.2% Triton X-100, pH 5.8, with a flow rate of 1 ml/min. (33). Fractions containing [35S]sulfate were collected and quantified by scintillation counting and subsequently pooled. Pooled fractions were further analyzed by size exclusion chromatography on an S-200 column (10 mm × 300 mm, flow 0.4 ml/min, Superdex, Amersham Bioscience) in 4 M guanidine hydrochloride, 50 mM NaOAc, 0.2% Triton X-100, pH 5.8 to separate the PGs by their hydrodynamic size. Separated PGs were collected in 0.4-ml fractions and aliquots were quantified by scintillation counting.

**COLABELED MOLECULES ([35S] AND [3H]).** The cell media was separated in a serial chromatography step. First, the samples were applied to ion-exchange chromatography (TSK gel, DEAE-5PW, 7.5 mm × 7.5 cm, Tosoh Bioscience, 50 mM phosphate buffer, pH 7.0) where the negatively charged PGs bind to the column and other molecules were washed. After a 20-min wash period (in same buffer as above), the proteins bound to the ion-exchanger were eluted by a step increase of NaCl concentration from 0 to 1 M to a size-exclusion column (Bio Sep-SEC-S3000, 300 × 7.8 mm, in 50 mM phosphate buffer, pH 7.0) at a flow rate of 0.5 ml/min. Separated PGs were collected in 0.5-ml fractions and aliquots were quantified by scintillation counting.

The size-exclusion columns were calibrated by using the LMW and HMW kits from Amersham Bioscience.

**Size Characterization of GAG Chains**

We treated the colabeled PG fractions with alkali-borohydride (1 M NaBH4 and 0.2 M NaOH) to cleave the binding between the GAG chains and the core proteins. The reaction was stopped by adding an equal volume of 0.2 M NaOH. GAG chains were precipitated (95% ethanol) and then separated on a size-exclusion column (Superdex 75, 10 mm × 30 cm, Pharmacia, in 4 M Guanidine/HCl, 0.2% Triton X-100, 50 mM NaOAc, pH 5.8) at flow rate of 0.4 ml/min. Separated GAG chains were collected in 0.4-ml fractions and quantified by scintillation counting.

**Statistics**

Results are presented as means ± SE, and differences were tested using the Student’s t-test pair design.

**RESULTS**

**Glomerular Endothelial Cell Culture**

The glomerular cells started to appear after approximately 1 wk, small colonies of different morphology of which about 1/5 was of endothelial origin. The cells were of endothelial origin as judged by light microscopy, by endocytosis of the endothelial cell-specific marker Dil-Ac-LDL, and by expression of the

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**Fig. 1.** Characterization of the human glomerular endothelial cells. A: primary human glomerular endothelial cells visualized by light microscopy. B: staining of glomerular endothelial cells by the endothelial-specific lectin Ulex europaeus visualized by fluorescence microscopy. C and D: endothelial-specific marker Dil-Ac-LDL endocytosed by glomerular endothelial cells visualized by fluorescence microscopy.
endothelial cell-specific lectin Ulex europaeus agglutinin as observed by fluorescence microscopy (Fig. 1). RT-PCR revealed that the cells expressed endothelin-1 (data not shown). No significant difference in number of cells or amount of RNA and protein was seen between the two groups after the experiment, confirming that there is no toxic effect by puromycin aminonucleoside at the concentration used (26).

**Gene Expression Analysis**

**Core proteins.** The following PGs were tested in the TaqMan real-time PCR analysis system and were found to be expressed in the human glomerular endothelial cells: syndecan, versican, glypican, perlecan, decorin, and biglycan (Table 1). The expression of versican was downregulated by 37 ± 6% (mean ± SE) in the PAN-treated group compared with the control (P < 0.001, n = 8). We could not see any significant difference in expression between the groups (control and PAN) for the other core proteins analyzed (Fig. 2A).

**Transferases.** Enzymes important for the degree of sulfation and the length of the GAG chains were also analyzed by real-time PCR. In galactosaminoglycan assembly, chondroitin 4-O-sulfotransferase was downregulated by PAN by 34 ± 7% (P < 0.01, n = 8) but not the 6-O-sulfotransferase. In addition, PAN downregulated the chondroitin synthase 1 by 25 ± 6% (P < 0.05, n = 6). Two of the GAG enzymes important for the heparan sulfate (HS)-biosynthesis were also affected: HS-ST6 (68 ± 4.5% of control) and HS-3-OST1 (65.5 ± 5% of control, P < 0.01, n = 6; Fig. 2B). The other enzymes tested (CS-6-O-ST, NDST, EXT-2, and HST-2) seemed to be unaffected by the treatment.

**Efficiency.** Validation of amplification efficiency was made for every primer/probe set and was calculated for each run from the corresponding standard curve, see Table 3. By using the standard curve equation [instead of comparative Ct (ΔΔCt) relative quantification method], we correlate for the differences in reaction efficiency between the target gene and the endogenous control gene.

**Protein Expression by Western Blot Analysis**

Syndecan, versican, and perlecan were expressed by the human glomerular endothelial cells as revealed by Western immunoblot analysis (Fig. 3). The versican antibody detects the V0 and V1 splice variants (∼370 and ∼260 kDa). When comparing the density of the enzyme-digested samples blotted for versican, we observed a 25% lower intensity in the PAN-treated cells compared with control. Syndecan (90 kDa) and perlecan (>400 kDa) were unaffected by the PAN treatment, a result in line with mRNA data.

**PG Synthesis in the Culture Media**

**Sulfate labeling.** The extracellular PGs in the conditioned medium from both control and PAN-stimulated cells were separated from other extracellular matrix molecules by charge density on a DEAE column. As shown in Fig. 4A, the total amount of labeled sulfate-containing PGs was drastically lower in the PAN-treated group compared with control (reduced by 60%). Further analysis of the peak fractions by size exclusion chromatography revealed that the two groups contained similar type of molecules. The PGs in the media seemed to be very large, 250–450 kDa, which suggests versican and/or perlecan. Colabeling with sulfate and tritiated glucosamine. Extracellular PGs in the conditioned medium from both control and PAN-stimulated cells were separated by both charge (on a DEAE column)- and by size-exclusion chromatography. As shown in Fig. 4B, the amount of large (>55 Å), sulfate-containing PGs was lower in the PAN-treated group compared with control (reduced by 40%). When measuring 3H-labeled GAG chains, we saw a reduction of 50% after PAN treatment (Fig. 4B).

**Size Characterization of the GAG Chains**

When analyzing the size of the GAG chains by size-exclusion chromatography, we could see a difference in the elution profile after PAN treatment. For the control cells, 25% of the GAG chains were in the size range of ∼40 kDa or greater, whereas the activity in the same fractions for PAN-treated cells was almost undetectable. In the smaller size ranges (∼14 kDa), we saw a delayed peak for the PAN-treated cells compared with control cells, indicating a reduced length of the GAG chains (Fig. 5).

**DISCUSSION**

Our study is the first to describe that the human glomerular endothelial cells (HGEC) express several different classes of PG core proteins. We have found that 1) HGECs express mRNAs for several PGs: syndecan, versican, glypican, perle-
can, decorin, and biglycan (both heparan and chondroitin sulfate containing). In addition, we showed at the protein level that HGECs synthesize and export syndecan, perlecan, and versican core proteins. The secreted PGs bind to structures and receptors situated on the cell-surface and they also interact with the GAG chains of other PGs (20, 51). Turley and Roth (49) showed in 1980 that there is an interaction between carbohydrate chains of hyaluronate and chondroitin sulfate. 2) The nephrosis-inducing agent PAN (18, 37) decreased both the mRNA expression (by 37%) and the protein expression (by 25%) of versican compared with control. Versican belongs to the family of hyaluronan-binding PGs that is collectively termed “hyalectans,” and these PGs are extracellular matrix components (20, 30). Pericellular matrix involvement involves the interaction of versican with several binding proteins and can be stimulated by growth factors such as PDGF and TGF-β1 (51). A decrease in versican could therefore result in a decrease of the pericellular matrix hence reducing the net negative charge of the cell-surface coat and/or the glomerular basement membrane. 3) In addition, PAN affected the enzymatic machinery behind the biosynthesis of chondroitin sulfate along the galactosaminoglycan biosynthetic pathway. Among the effects seen was a downregulation of the 4-O-sulfotransferase (CSST4) by 34%. CSST4 adds sulfate to the fourth carbon position on GalNac residues. This downregulation will reduce the total negative charge of versican, as no compensatory upregulation by other sulfotransferases (i.e., CS-6-O-sulfotransferase) is seen. Because versican normally carries CS, sulfotransferases acting on dermatan sulfate GAGs can be disregarded. It should, however, be noted that 4-O-sulfation on GalNac is a prerequisite for dermatan sulfate stability by locking the L-iduronic acid in its energetically unfavorable L-form (29). This prevents the iduronic acid to switch back to its C-5 epimer in chondroitin sulfate, glucuronic acid (GlcA).

With this in mind, it is also likely that decorin and biglycan (small PGs capable of carrying either dermatan or chondroitin sulfate) in this case mostly carry chondroitin sulfate chains (10, 20). Moreover, PAN downregulated the enzyme CS synthase, responsible for the elongation of the GAG chains, by 25%. Thus PAN induced a loss of negative charge by versican core protein downregulation, a loss of negative charge per versican molecule, and possibly a stereochemical alteration of the CS/DS content of the endothelial matrix. This indicates a total deorganization of the matrix, where the alteration of biological function can be expected to be severe in the context of tissue function. 4) Both syndecan and glypican are membrane bound and may be important for the structure of the GEC surface coat by acting as anchors for the pericellular gel structure (3). Glypican is heavily involved in cell-to-cell contact and morphogenesis (9). As described above, we could not detect changes in glypican expression, consistent with our observation that PAN (in this concentration) did not affect cell morphology. Syndecans have, in addition to many other functions, similar characteristics for cellular behavior including cell signaling (44). Similar to glypican, the syndecan expression was not affected on the core protein level. However, the observed downregulation of HS-ST6 and HS-ST3 could result in a decreased charge density on the immediate cell surface of the membrane bound PGs as well. It may also have biological importance for the binding of cytokines (such as FGF) in similar ways earlier reported (27). 5) The analysis of the cell media revealed that the glomerular endothelial cells produce a large amount of sulfate-containing GAGs. Versican and/or perlecan were the major core proteins found in the media. PAN reduced the total amount of sulfate-labeled PGs by 60%. When analyzing the larger molecules (>55 Å), we saw a 40%
reduction of sulfate and a 50% decrease of tritiated glucosamine. This reduction is caused by loss in the amount of sulfate groups and by a reduced length of GAG chains due to downregulation of the enzymes mentioned above in combination with the decrease in versican expression. 6) Puromycin did not seem to affect the mRNA expression of the core proteins syndecan, glypican, decorin, biglycan, or perlecan. Neither did PAN affect the enzymes CS-6-O-ST, NDST, EXT-2, and HST-2.

A decreased synthesis of GAGs could increase the permeability of the layer covering the fenestrae and hence increase the sieving of macromolecules (17). In fact, effects of PAN on glomerular endothelial cell fenestrations have been described earlier by Avasti and Evan (1) but they reported the fenestrae to be decreased in size and number during PAN-induced nephrosis, as seen by TEM/SEM.

The role of glomerular endothelial cells for the charge and size selectivity of the filtration barrier in the kidney has been argued for several years. Recent functional studies from our laboratory (18, 22) are in qualitative agreement with the classical studies of a significant negative charge barrier, albeit with a lower charge density (50 vs. 100–150 meq/l) (47). However, when studying the charge barrier in vitro, experiments are conducted mainly on mesangial and epithelial cells to measure their production of negative charged molecules, i.e., PGs and glycoproteins (11, 13). Furthermore, isolated glomerular basement membranes showed some size but lacked charge selectivity in vitro (4), which has been taken as evidence of its limited role in the glomerular charge barrier. However, the isolation procedure may change the composition of the basement membranes and their permeabilities seem to partially depend on the hydrostatic pressures applied (38). In any case, knockout mice lacking the heparan sulfate chains of perlecan (the principal PG of the GBM) do not develop any kidney malfunction, such as proteinuria (39). There are other components, such as agrin, in the GBM which also might be of importance for the charge-selective properties of the barrier.

Anionic sites have also been demonstrated on glomerular epithelial cells (12). The importance of the GAGs is illustrated in a study where the gene for one of the enzymes responsible for site-specific sulfation of heparan sulfate (HS 2-O-sulfotransferase) was knocked out. The mice did not develop any kidneys, as a result from failure of ureteric bud branching and mesenchymal condensation, and they died shortly after birth (5).

It is already known that endothelial cells express different PG core proteins such as versican (6), perlecan (50), syndecan (3, 19), biglycan (23), glypican (3), and decorin (21). These

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**Fig. 4.** A: total cell media activity of incorporated [35S]sulfate in cpm for control and PAN-treated groups after ion-exchange (DEAE) chromatography. B: cell media activity of incorporated [3H]sulfate and [3H]glucosamine for large molecules (>55 Å) after ion-exchange and size-exclusion chromatography. The graph illustrates PAN-treated cells compared with control.

**Fig. 5.** Activity of incorporated [3H]glucosamine in GAG chains after alkali-borohydride treatment when separated by size-exclusion chromatography. The elution profile was changed after PAN treatment with a delayed peak, indicating shorter length of the GAG chains.
studies were made in macrovascular endothelial cells, mainly from the aorta and the umbilical vein. However, there seem to be differences between endothelial cells originating from different vasculature (6, 53). The only study to describe human glomerular endothelial cell PG production is from Schaefer et al. (42) where they showed an expression of biglycan by using in situ hybridization. On the other hand, they did not find any expression of decorin. The present paper is therefore the first to demonstrate that glomerular endothelial cells produce several different classes of PGs.

We conclude that human GECs produce several different classes of PGs. The nephrosis inducing agent PAN downregulates the expression of only one core protein (versican) but has severe effects on foremost key galactosaminoglycan chondroitin sulfate transferases and some sulfation-linked effect on glucosaminoglycan heparan sulfate biosynthesis. The overall effect in this context is less sulfate groups, shorter GAG chains, different classes of PGs. The nephrosis inducing agent PAN downregulates the expression of decorin. The present paper is therefore the first to describe human glomerular endothelial cell proteoglycan–regulation by transforming growth factor-β, in cultured bovine aortic endothelial cells exhibiting angiogenesis in vitro. Exp Cell Res 203: 395–401, 1992.


ACKNOWLEDGMENTS
The Department of Urology at Sahlgrenska University Hospital supplied the tissue samples for which we are most grateful.

GRANTS
This study was supported by Swedish Medical Research Council Grants 9898, 14764, and 4531, the IngaBritt and Arne Lundberg Research Foundation, the National Association for Kidney Diseases, Sahlgrenska University Hospital Grant LUA-511733, Sahlgrenska University Hospital Foundation, The Swedish Heart and Lung Foundation, and AstraZeneca.

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