No Change in Glomerular Heparan Sulfate Structure in Early Human and Experimental Diabetic Nephropathy*

Received for publication, February 17, 2006, and in revised form, July 13, 2006 Published, JBC Papers in Press, August 1, 2006, DOI 10.1074/jbc.M601552200

Jacob van den Born‡, Brenda Pisa‡, Marinka A. H. Bakker‡, Johanna W. A. M. Celie§, Carin Straatman‡, Steven Thomas¶, Gian C. Viberti‡, Lena Kjellen‖, and Jo H. M. Berden‡

From the ‡Department of Nephrology, Radboud University Medical Center Nijmegen, P. O. Box 9101, 6500 HB Nijmegen, The Netherlands, the §Department of Molecular Cell Biology and Immunology, VU University Medical Center, P. O. Box 7057, 1007 MB Amsterdam, The Netherlands, the ¶Department of Diabetes, Endocrinology, and Internal Medicine, Guy’s Hospital, St. Thomas Street, London SE1 9RT, United Kingdom, and the ‖Department of Medical Biochemistry and Microbiology, Uppsala University, Box 582, SE-751 23, Uppsala, Sweden

Heparan sulfate (HS) proteoglycans are major anionic glycoconjugates of the glomerular basement membrane and are thought to contribute to the permeability properties of the glomerular capillary wall. In this study we evaluated whether the development of (micro)albuminuria in early human and experimental diabetic nephropathy is related to changes in glomerular HS expression or structure. Using a panel of recently characterized antibodies, glomerular HS expression was studied in kidney biopsies of type I diabetic patients with microalbuminuria or early albuminuria and in rat renal tissue after 5 months diabetes duration. Glomerular staining, however, revealed no differences between control and diabetic specimens. A significant (p < 0.05) ~60% increase was found in HS N-deacetylase activity, a key enzyme in HS sulfation reactions, in diabetic glomeruli. Structural analysis of glomerular HS after in vivo and in vitro radiolabeling techniques revealed no changes in HS N-sulfation or charge density. Also HS chain length, protein binding properties, as well as disaccharide composition did not differ between control and diabetic glomerular HS samples. These results indicate that in experimental and early human diabetic nephropathy, increased urinary albumin excretion is not caused by loss of glomerular HS expression or sulfation and suggest other mechanisms to be responsible for increased glomerular albumin permeability.

One of the first signs of diabetic nephropathy (DNP) is a discrete increase in urinary excretion of albumin called microalbuminuria (1, 2). In this early phase of DNP mainly the charge-dependent permeability of the glomerular capillary wall seems to be affected, while during progression to overt proteinuria also the size-dependent permeability becomes disturbed (3). These findings suggest an early loss of glomerular basement membrane (GBM) charge with a subsequent increase in pore size. This charge-dependent permeability of the GBM is probably related to the presence of anionic constituents of the GBM, mainly heparan sulfates (HS), the glycosaminoglycan side chains of heparan sulfate proteoglycans (HSPGs) (4). Some years ago, we identified agrin as a major HSPG present in the GBM (5, 6), using antibodies toward either the core protein or the HS polysaccharide side chains (7–9). The relevance of HS for the charge-dependent permeability is illustrated by several observations: (i) enzymatic removal of HS from the GBM leads to albuminuria (10); (ii) an acute selective albuminuria was induced in rats by a monoclonal antibody to HS (4); (iii) a reduction of HS expression in the GBM was documented in several human and experimental proteinuric glomerular diseases, which inversely correlated with proteinuria (11, 12).

In overt DNP, the expression of HS in the GBM was decreased and correlated with proteinuria (13, 14). However, these studies were performed in advanced stages of DNP. In a rat diabetes model resembling early human DNP, we documented a significant loss in HS-related anionic sites in the GBM; however, without loss of GBM HS (15, 16). This suggested undersulfation of GBM HS as an early event in DNP (17). In 1989 Deckert and colleagues (18) formulated the hypothesis that decreased HS expression and/or sulfation was an early event in DNP and microangiopathy. Our previous findings prompted us to investigate the eventual involvement of GBM HS in the onset of albuminuria in experimental and human insulin-dependent diabetes mellitus (IDDM). To this end we induced diabetes in the rat by streptozotocin. After 5 months of diabetes duration, the presence and structure of glomerular HS was analyzed using different approaches including immunohistochemistry with anti-HS mAbs, radiolabeling techniques, and measurement of HS synthesizing enzymes. Finally, HS expression was evaluated in human kidney biopsies of IDDM patients with microalbuminuria and early proteinuria without hypertension.

We now show that the development of albuminuria in early experimental and human DNP is not associated by evident changes in GBM HS expression, sulfation, or structure. These results indicate that glomerular HS change is not an initial event leading to increased urinary albumin excretion in incipi-
ent DNP, although HS loss might develop in later stages of the disease.

**EXPERIMENTAL PROCEDURES**

**Animals**—Diabetes was induced in male Wister-Münich rats by a single intravenous injection of 55 mg of streptozotocin/kg of body weight as described previously (15, 16). Sex- and age-matched rats served as controls. Diabetic rats received three times a week a low dose (1–1.5 IU) of bovine insulin (Ultralente; Novo, Copenhagen, Denmark) to maintain blood glucose between 20 and 25 mmol/liter. Rats were followed for their body weight, blood glucose, urine production, and albuminuria as described previously (15, 16). After 5 months six control and six diabetic rats were sacrificed, and the kidneys were removed and weighed. A small piece of renal tissue was snap-frozen in liquid nitrogen for immunohistology (see below), and subsequently glomeruli of individual rats were isolated by differential sieving. Glomeruli of each rat were divided into two equal portions, one portion was subjected to detergent treatment to measure NDST enzymatic activity, and the second portion was used for structural HS analysis (see below). In separate groups of rats (four control and four diabetic rats), after the same duration of diabetes, in vivo radiolabeling of proteoglycans was achieved by two intraperitoneal injections of [35S]sulfate (ICN Radiochemicals), with a time interval of 4 h. Four hours after the last injection, animals were sacrificed, kidneys were removed, and glomeruli of individual rats were isolated by differential sieving. All animal studies were approved by the ethical committee on experimental animals of the Radboud University Medical Center in Nijmegen and were in agreement with the National Institutes of Health guide for the care and use of laboratory animals.

**Human Renal Tissues**—Kidney biopsies were obtained from the ESPRIT study (19), in which the effect was evaluated of enalapril, nifedipine retard, or placebo on the progression of diabetic nephropathy in normotensive insulin-dependent diabetic patients with increased urinary albumin excretion. Kidney biopsies were taken at the start of the study (n = 54) and after 3 years of treatment (n = 35). From 19 biopsies taken after treatment, paraformaldehyde-fixed, paraffin-embedded tissue was available for this study. Eight of the 19 biopsies did not meet the inclusion criteria (at least 3 glomeruli/section), thus 11 biopsies were evaluated. Control human renal tissue from six non-diabetic individuals was obtained from the distant pole of kidneys removed for localized malignancy. These control tissues were fixed and embedded identical to the biopsies of the diabetic patients. A schematic outline of the experimental approach (both rat studies and human biopsies) is given in Fig. 1.

**Immunohistology**—Indirect immunofluorescence microscopy on 2-μm rat kidney cryostat sections was performed as described (11, 16). Mab 3G10 (Seikagaku Corp., Tokyo, Japan), reacting with heparitinase-created HS-stubs (independent of the HSPG core protein specificity) was used as a pan-HS marker. Digestion of the tissue sections (before 3G10 staining) was done for 1 h at 37 °C, 0.25 unit/ml heparitinase I (Sigma, EC 4.2.2.8). Mouse anti-HS mAbs 10E4 and HepSS1 were commercially obtained (Seikagaku) and have been recently characterized in detail (20). mAb 865 recognizes the Escherichia coli capsular K5 polysaccharide and unsulfated domains in HS (8). Mouse anti-HS mAbs JM-13 and JM-403 have also been characterized (7, 9, 20). Control experiments in which the fluorescein isothiocyanate-labeled secondary antibodies were applied to the sections without prior primary antibody incubation were consistently negative. Sections were embedded in Vectashield (Vector Laboratories Inc., Burlingame, CA) and examined on a Zeiss Axioskop microscope equipped for fluorescence microscopy.

Indirect immunoperoxidase staining was performed on human renal tissues that were sectioned at 2 μm, deparaffinized and rehydrated. Endogenous peroxidase activity was quenched for 30 min by 20% H2O2 in phosphate-buffered saline. Antigen retrieval was performed for 7 min at 37 °C with 0.1% Pronase type XIV (Sigma). Subsequently, sections were preincubated with 20% normal rabbit serum. Agrin core protein was stained with goat polyclonal antibody BI-31 (21), followed by peroxidase-conjugated rabbit anti-goat IgG (Dako). HS was stained by mAb JM-403, followed by biotinylated rabbit anti-mouse IgM (Dako) and peroxidase-conjugated streptavidin biotin complex (Dako). Diaminobenzidine tetrahy-
Heparan Sulfate and Diabetic Nephropathy

drochloride was used as the substrate. Finally, a counterstaining was performed with hematoxylin. Sections were dehydrated and mounted in Depex mounting medium (BDH). The same person, using identical conditions on a single day, performed all staining procedures for each given antibody. Specificity of the staining was controlled using irrelevant goat and mouse antibodies. Slides (rat and human renal tissues) were numbered randomly and examined by two independent observers without knowledge of the sample origin.

Partial HS Analysis—One portion of the isolated glomerular of control and diabetic rats were pooled separately. HS was isolated according to standard procedures, which comprises papain digestion, DEAE-Sephaloc ion-exchange chromatography, and chondroitinase ABC treatment followed by Mono Q fast protein liquid chromatography. Both HS samples were subjected to two different labeling procedures. Half of the material was selective cleaved at the N-sulfated GlcN units by nitrous acid at pH 1.5 followed by end labeling of the fragments by NaB\(^{3}\)H\(_{4}\) (22, 23). The resultant radiolabeled oligosaccharides were separated on a column (1 × 140 cm) of Bio-Gel P-10 fine (Bio-Rad) in 0.5 M ammonium hydrogen carbonate. From the elution pattern the percentage of N-sulfation can be calculated (34). The HexA-\([\text{3H}]\)aMan\(_{4}\) disaccharide fractions were pooled and analyzed for their composition by ion exchange HPLC using a Whatman Partisil-10 SAX column (4.6 × 250 mm). The nonsulfated disaccharides, GlcA-aMan\(_{4}\) and IdoA-aMan\(_{4}\), were not resolved in this procedure and were instead separated by descending paper chromatography (25). The second part of the glomerular HS samples was partly deacetylated for 1 h by hydrazinolysis, followed by N-acetylation using \([\text{3H}]\)acetic anhydride (26, 27). These radiolabeled HS preparations were used to perform protein binding experiments (see below) and to estimate the length of the chains by Sephacryl S-300 high resolution (Pharmacia, Uppsala, Sweden) gel chromatography, 1 × 70-cm column, in 0.5 m NaCl.

In vivo \([\text{35S}]\)sulfate-radiolabeled glomerular HS (in a separate group of four control and four diabetic rats) was isolated according to standard procedures. HPLC DEAE-Sephaloc ion-exchange chromatography was performed using a linear gradient from 0 to 1.5 m LiCl.

Protein Binding of Glomerular HS—FGF-2 and antithrombin III were immobilized to cyanogen bromide-activated Sepharose. \([\text{3H}]\)Acetylated glomerular HS (10,000 cpm) from either control or diabetic rats was loaded on the column, washed with phosphate-buffered saline, and eluted by increasing concentrations of NaCl. Radioactivity was counted by scintillation counting. As a positive control the binding of \([\text{3H}]\)heparin to FGF-2-Sepharose or antithrombin III-Sepharose was included. As a negative control we included the binding of \([\text{3H}]\)HS and \([\text{3H}]\)heparin toward BSA-Sepharose, which was consistently negative.

In a second approach the binding of glomerular HS to FGF-2 was studied in a filter assay as described (28). Radioactivity of the eluate was measured after addition of 10 ml of Optiphase “HiSafe 3” (Wallac, Breda, The Netherlands).

N-Deacetylase Assay—To measure the enzymatic N-deacetylase activity of the NDST enzymes, the second portion of the individual glomerular samples was subjected to detergent treatment. Glomeruli were homogenized in 5 µl/mg glomeruli of 50 mM Tris, pH 7.4, containing 2 mM EDTA, 1% Triton X-100 and a mixture of protease inhibitors (Complete™, Roche Applied Science). The homogenates were centrifuged for 15 min at 13,000 rpm at 4°C, and the supernatant was collected and diluted 1:1 in glycerol and stored in −20°C until use. Protein concentration in the glomerular lysates was determined by the method of Lowry (29). The N-deacetylase assay, which we described before (30), is based on the recognition of N-deacetylated K5 by mAb JM-403. The enzyme-linked immunosorbent assay signal is proportional to the number of N-deacetylated GlcN units created in the K5 polysaccharide and thus proportional to the NDST N-deacetylase activity. Glomerular samples were all measured at a protein concentration of 200 µg/ml.

Statistics—Where applicable, data are presented as median values (and range). Significance (p < 0.05) was tested by the unpaired Mann Whitney U test.

RESULTS

Development of Diabetic Nephropathy in STZ Diabetic Rats—After a diabetes duration of 5 month an increased urinary albumin excretion developed and enlargement of the kidneys, which are both characteristic for diabetic nephropathy (Table 1). The diabetic animals were hyperglycemic during the entire study period as controlled by weekly blood glucose monitoring. The blood glucose values in Table 1 correspond to the blood glucose levels measured just before the animals were killed. Table 1 also shows that the diabetic rats gained less weight compared with control rats. Light microscopy demonstrated a moderate increase of the mesangial matrix in the glomeruli of most diabetic rats as evidenced previously (16) (data not shown).

Immunohistology Using Anti-HS Antibodies—As evidenced by Fig. 2A, 3G10 staining of control renal tissue (recognizing all HS stubs after heparitinase) revealed a brilliant staining of the glomerular basement membranes along with staining of the mesangial areas and Bowman's capsule around the glomeruli. Exactly the same staining pattern was observed in the diabetic glomeruli indicating a comparable HS expression within these particular structures (Fig. 2B). JM403 and mAb 865 recognize particularly glomerular basement membrane HS (Fig. 2, E and F), while another set of mAbs (10E4 and HepSS1) do not stain the glomeruli at all but show binding to Bowman's capsule and the basal laminae of vascular smooth muscle cells (Fig. 2, C and D). mAb JM13 shows a weak undefined staining within the glomerulus, along with light staining of Bowman's capsule and a variety of tubular basement membranes (data not shown). Taking into account the limitations of this immunohistochem-

### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Diabetic rats (n = 6)</th>
<th>Control rats (n = 6)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>264 (246–311)</td>
<td>396 (322–433)</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>3.66 (2.60–3.97)</td>
<td>2.53 (2.15–3.12)</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Blood glucose (mmol/l)</td>
<td>21.6 (19.2–24.2)</td>
<td>3.8 (3.6–4.0)</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>Diuresis (ml/24 h)</td>
<td>162 (115–205)</td>
<td>15 (12–24)</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>Albuminuria (mg/24 h)</td>
<td>36 (2–64)</td>
<td>3 (1–9)</td>
<td>&lt;0.03</td>
</tr>
</tbody>
</table>

Downloaded from www.jbc.org at Radboud Universiteit Nijmegen on May 16, 2012
ical approach, no obvious differences could be observed in staining patterns between diabetic and control rat kidneys with any of the anti-HS mAbs.

Partial Analysis of Glomerular HS—Rats were injected with [35S]sulfate to get in vivo incorporation into proteoglycans. Isolated glomerular HS of individual rats were subjected to HPLC DEAE-Sephacel ion-exchange chromatography. Profiles of both control and diabetic glomerular HS (Fig. 3) fail to reveal any differences in HS charge. Both control and diabetic glomerular HS elute at fraction 38, which corresponds to a concentration of ~1.05 M LiCl, and is identical to the elution position of standard HS from bovine kidney (0.84 sulfate group/disaccharide).

For the HS analysis partial N-deacetylation was followed by N-acetylation using [3H]acetic anhydride. Samples were subjected to Sephacryl S-300 high resolution gel filtration. Elution profiles are shown in Fig. 4A. Both control and diabetic glomerular HS were indistinguishable and eluted at a position between HS from bovine kidney and heparin, indicating an molecular mass of 18–20 kDa. Nitrous acid pH 1.5 deamination products obtained after reduction with NaB₃H₄ (100,000 cpm) were size separated on a Bio-Gel P10 fine column. This resulted in nearly identical size distribution of the obtained HS oligosaccharides of control and diabetic glomerular samples (Fig. 4B). N-Sulfation was 44% in the control and 47% in the diabetic sample. We next pooled the disaccharide peak from the Bio-Gel P10 column and analyzed the disaccharide composition by ion exchange HPLC (Fig. 4C). These disaccharides originated from the contiguous N-sulfated blocks of the HS chains. The three mono-O-sulfated disaccharides display a composition, which is very similar to HS, isolated from bovine kidney, showing a dominant IdoA(2-OSO₃)-aManR peak both in control and diabetic N-sulfated blocks. Interestingly, we noticed a most unusual pattern with two di-O-sulfated disaccharides. The first one, at 123–124 min, is most likely IdoA(2-OSO₃)-aManR(6-OSO₃). The more retarded component at 128–129 min is not GlcA-aManR(3,6-diOSO₃), since it resists digestion with β-glucuronidase, which, as a control, eliminates the peak of GlcA-aManR(6-OSO₃). Instead, we believe this is IdoA(2-OSO₃)-aManR(3-OSO₃), which has been described earlier in glomerular basement membrane HS (31, 32). No obvious changes are observed in the distribution of the various disaccharides between control and diabetic samples (Fig. 4C), although the most retarded 3-OSO₃ disaccharide might be a bit increased in diabetic HS. GlcA-aMan₉ form the large majority of the non-sulfated disaccharides both in diabetic and control samples as revealed by descending paper chromatography (data

FIGURE 2. Indirect immunofluorescence with anti-HS mAbs 3G10 (A and B), 10E4 (C and D), and mAb 865 (E and F) on renal tissue from control (A, C, and E) and diabetic (B, D, and F) rats. Glomerular staining depends on the presence or absence of particular HS motifs. Note the lack of differences between control and diabetic tissues. Original magnification: ×250.

FIGURE 3. Determination of glomerular HS charge. Samples of radiolabeled glomerular HS were obtained after in vivo incorporation of [35S]sulfate followed by glomerular sieving and HS isolation and were subjected to HPLC DEAE-Sephacel anion exchange chromatography. Both control (open circles) and diabetic (black circles) HS eluted at fraction 38 (corresponding to ~1.05 M LiCl). Elution positions of standards K5 polysaccharide (no sulfates), HS from bovine kidney (0.84 sulfate/disaccharide), and heparin (~2.5 sulfates/disaccharide) are indicated by arrows.
TABLE 2

Protein binding characteristics of control and diabetic glomerular HS in comparison with heparin. IC_{50} represent the molarity of NaCl needed to elute 50% of bound polysaccharides from the affinity column or nitrocellulose filter.

<table>
<thead>
<tr>
<th></th>
<th>Control rats</th>
<th></th>
<th>Diabetic rats</th>
<th></th>
<th>Heparin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% binding</td>
<td>IC_{50}</td>
<td>% binding</td>
<td>IC_{50}</td>
<td>% binding</td>
</tr>
<tr>
<td><strong>Affinity column</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATIII</td>
<td>5.4</td>
<td>ND</td>
<td>5.3</td>
<td>ND</td>
<td>23.1</td>
</tr>
<tr>
<td>FGF-2</td>
<td>86.3</td>
<td>0.51</td>
<td>84.4</td>
<td>0.51</td>
<td>92.5</td>
</tr>
<tr>
<td><strong>Filter assay</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF-2</td>
<td>49.7</td>
<td>0.38</td>
<td>51.9</td>
<td>0.38</td>
<td>54.0</td>
</tr>
</tbody>
</table>

not shown). These analytical data indicate no apparent diabetes-induced changes in glomerular HS charge, length, N-sulfation, and composition of the disaccharides within the N-sulfated domains.

**Protein Binding of Glomerular HS**—As can be seen in Table 2 most glomerular HS failed to bind to antithrombin III. The small fraction that bound however (≈5.5%) eluted predominately after the addition of 0.5 M NaCl. No differences were observed between diabetic and control glomerular HS. As a control [3H]heparin bound for ≈23% to the column, which indicates functionality of the immobilized antithrombin III. Glomerular HS was also tested for binding to FGF-2. Both HS samples bound ≈85% to FGF-2. No differences could be observed between both samples; 50% elution was ≈0.5 M NaCl. As a control we loaded [3H]heparin to FGF-2 column, which bound for 92.5% and eluted for 50% at a NaCl concentration of ≈0.8 M (Table 2). These results were confirmed using a filter binding assay (Table 2).

**N-Deacetylase Assay**—Half of the sieved glomeruli were used for measurement of the enzymatic N-deacetylase activity of the NDST enzymes. Fig. 5A shows a linear increase in enzyme-linked immunosorbent assay signal, proportional to the amount of glomerular protein of a control rat. Fig. 5B shows a significant increased N-deacetylase activity in the diabetic glomeruli compared with the control glomeruli (p < 0.05). The same significance was also found when the results were expressed per 10,000 glomeruli (data not shown).

**Early Human Diabetic Nephropathy**—In control human kidneys the GBM displayed a linear staining both for agrin core protein (ab Bl-31; Fig. 6A) as well as for HS (visualized by mAb JM-403; Fig. 6C). The mesangial matrix is also positive for agrin and HS. Both antibodies also stained the GBM in an identical brilliant linear fashion in the biopsies from all diabetic patients, irrespective of treatment or whether they are micro- or macroalbuminuric (Fig. 6, B and D). The expanded mesangium is also clearly positive by both antibodies. We could not find any differences in GBM staining pattern and intensity between control and diabetic individuals. This results indicate that in normotensive type 1 diabetic patients the development of early diabetic nephropathy, characterized by the presence of albuminuria, is not associated with apparent change in GBM HS.

**DISCUSSION**

In this study we show that the development of microalbuminuria and early proteinuria in experimental and human DNP is not associated with changes in the expression pattern or structure/sulfation of glomerular HS.

In our experimental approach we visualized HS by recently characterized anti-HS mAbs, we analyzed glomerular HS structure by a number of different analytical techniques, we measured glomerular N-deacetylase enzymatic activity, and we compared STZ-induced diabetes in the rat with early human diabetic nephropathy. Despite this extensive approach, only one significant diabetes-induced change was observed, namely a ≈60% increase in N-deacetylase enzymatic activity, which, however, was not accompanied by a change in glomerular HS N-sulfation. A number of publications describe a diabetes-induced inhibition of HS NDST and/or HS N-sulfation, with conflicting results (33–40). Our data indicate that determination of enzyme activity does not safely predict changes in structure or sulfation of the HS polysaccharide.

Our study reveals two other interesting findings. First, mean sulfation of glomerular HS is ≈0.84 sulfate group/disaccharide. This is characteristic for a modest sulfated HS isoform and seriously questions the concept of electrostatic repulsion of albumin by glomerular HS charge. Second, our disaccharide analysis revealed a rather unusual disaccharide within the N-sulfated domains, namely IdoA(2-OSO_3)-aManR(3-OSO_3), which has been described earlier in GBM HS (32), and is documented to be reduced in diabetic GBM (31), which, however, is not found in our analysis. The presence of this unique disaccharide points toward the glomerular presence of a 3-O-sulfotransferase isoform, which generates this 3-O-sulfate adjacent to a 2-O-sulfated IdoA unit. A likely candidate would be 3-O-sulfotransferase-3 (41), whose expression in the kidney has been described. The significance of this rare HS sequence is not clear...
yet but might be related to some functional properties of the GBM.

Our findings bring about some fundamental questions about the increased albumin excretion in DNP. The development of DNP is associated with a well-documented disturbance in the charge-dependent permeability properties, characterized by selective loss of albumin and a diminished number of anionic sites in the GBM. In most studies these anionic sites are heparitinase digestible (14, 42), pointing toward the HS identity of these sites. However, the present study failed to show any diabetes-induced HS changes. Apparently, both the reduction in heparitinase-digestable anionic sites, as well as the increased albumin excretion might be achieved by other diabetes-related factors such as the non-enzymatic glycation of intrinsic GBM constituents or trapping of glycated serum proteins, thereby changing the three-dimensional architecture of the GBM and interfering with the staining of the HS-associated anionic sites and the permeability for albumin (43).

Concerning the intact GBM HS staining in early human DNP, we previously reported that the GBM HS staining was partly or completely lost in advanced DNP (13). Moreover, the loss of GBM HS stainability correlated with the rate of proteinuria. This was also found in a study of Vernier using cuprolinic blue to stain HS-associated anionic sites in the GBM of DNP in type I diabetes (14). We propose that loss of GBM HS might be secondary to the development of proteinuria and probably related to flattening and fusion of podocytic foot processes. Our data indicate that besides GBM HS other glomerular determinants are instrumental in glomerular charge-dependent permeability properties (44). In this respect, new insights in the cell biology of the glomerular podocyte, especially the unraveling at the molecular level of the slit diaphragms (45, 46), has intensified the general interest in the role of the podocyte in the permeability characteristics of the glomerular capillary wall under normal and diseased conditions, including diabetes (47, 48). A recent publication suggests high glucose-driven heparanase-1 expression in glomerular epithelial cells in culture (49). Although we did not include this enzyme in the present study, our analytical data, especially the unchanged HS chain length, do not support HS degradation in the STZ diabetic rats. Current ideas on the initiation phase of diabetic nephropathy indicate that hyperglyce-
Heparan Sulfate and Diabetic Nephropathy

References