In Vivo Degradation of Heparan Sulfates in the Glomerular Basement Membrane Does Not Result in Proteinuria

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Heparan sulfates (HS) are long, unbranched, negatively charged polysaccharides that are bound to core proteins. HS in the glomerular basement membrane (GBM) is reported to be important for charge-selective permeability. Aberrant GBM HS expression has been observed in several glomerular diseases, such as diabetic nephropathy and membranous glomerulopathy, and a decrease in HS generally is associated with proteinuria. This study, with the use of a controlled in vivo approach, evaluated whether degradation of HS in rat GBM resulted in acute proteinuria. Rats received two intravenous injections of either heparinase III to digest HS or neuraminidase to remove neuraminic acids (positive control). Urine samples were taken at various time points, and at the end of the experiment, kidneys were removed and analyzed. Injection with heparinase III resulted in a complete loss of glomerular HS as demonstrated by immunofluorescence staining using anti-HS antibodies and by electron microscopy using cupromeronic blue in a critical electrolyte concentration mode. In the urine, a strong increase in HS was found within 2h after the first injection. Staining for agrin, the major HS proteoglycan core protein in the GBM, was unaltered. No urinary albumin or other proteins were detected at any time point, and no changes in glomerular morphology were noticed. Injection of rats with neuraminidase, however, resulted in a major increase of urinary albumin and was associated with an increase in urinary free neuraminic acid. An increased glomerular staining with Peanut agglutinin lectin, indicative of removal of neuraminic acid, was noted. In conclusion, removal of HS from the GBM does not result in acute albuminuria, whereas removal of neuraminic acid does.


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intravenous injection of rats with the monoclonal anti-HS antibody JM403 resulted in a dosage-dependent increase in albuminuria (17). Second, in isolated rat kidneys or glomeruli that were perfused with the HS-degrading enzyme heparinase III or with charge-neutralizing protamine, native ferritin molecules (18,19) and BSA (19,20) were found to penetrate the GBM and reach the urinary space. However, in a study by Jeansson et al. (21), the role of HS on glomerular filtration was not obvious. It should be noted that in none of these studies thoroughly evaluated the removal or blocking of HS. Because it still is not clear whether HS plays a major role in the development of proteinuria, the aim of our study was to investigate in a controlled in vivo study the functional importance of this polysaccharide for glomerular permeability of proteins.

Materials and Methods

Experimental Setup

Before injection into the tail vein of rats, the enzymatic activities of heparinase III (E.C. 4.2.2.8; from Flavobacterium heparinum; molecular weight 73 kD; IBEX, MT-Royal, QU, Canada) and a2-3,6,8-neuraminidase (E.C. 3.2.1.18; from Vibrio cholerae; molecular weight 90 kD; Calbiochem, La Jolla, CA) were evaluated in situ. Rat cryosections (2 μm) were preincubated with heparinase III in 25 mM Tris (pH 8.0) or neuraminidase in 50 mM sodium acetate and 1 mM CaCl₂ for 2 h at 37°C. HS staining (see the Immunofluorescence Staining section) was abolished after pretreatment of cryosections with 0.02 IU/ml heparinase III, whereas complete removal of neuraminic acid was found using 0.04 IU/ml neuraminidase (Figure 1). On the basis of these results and taking into account that Wistar rats contain approximately 10 ml of plasma, 0.2 to 0.4 IU of heparinase III and 0.4 IU neuraminidase were administered intravenously. Using a protease fluorescence detection kit (Sigma, St. Louis, MO) according to the manufacturer’s protocol, we checked whether the heparinase III and neuraminidase solutions contained protease activity. No protease activity was detected in the enzyme solutions.

Male Wistar rats (250 to 300 g) were housed in the animal facilities of the Central Animal Facility of the Radboud University Nijmegen Medical Centre with free access to standard pelleted food and drinking water. The animal ethics board approved all animal experiments. Rats received two intravenous injections of 1 ml of PBS that contained the enzymes heparinase III or neuraminidase. The experiment was performed according to the schedule shown in Table 1. Briefly, rats were administered two injections (t = 0 h and t = 24 h) of heparinase III (0.2 IU) or PBS via the tail vein and killed 48 h after the first injection. Another set of rats were administered an injection of 0.4 IU instead of 0.2 IU of heparinase III and with a shorter interval between the injections (8 instead of 24 h). In addition, rats were administered an injection of neuraminidase (0.4 IU), which served as a positive control for the evaluation of our in vivo system, because this treatment resulted in proteinuria (22). The rats were killed 24 h after the first injection. Two rats were taken per experimental condition. For obtaining urine samples, rats were placed individually into metabolic cages immediately after the first injection. At the end of the experiment, blood was collected to obtain serum. Kidneys were removed and processed for immunofluorescence staining, general histology, and electron microscopy.

Immunofluorescence Staining

Frozen rat kidneys were sliced into 2-μm sections and stored at −20°C until use. After air-drying, cryosections were blocked with PBS that contained 2% (wt/vol) BSA and 0.05% (vol/vol) Tween-20 (blocking buffer) for 20 min. Cryosections were then incubated with phage display–derived anti-HS antibodies (produced in our laboratory [23–25] and recognizing sulfated HS domains) in blocking buffer for 1 h. As a negative control, an antibody from an irrelevant clone (MPB49) was used. This antibody, which is >95% identical to the antibodies used, does not stain kidney tissue sections. Bound antibodies were detected by incubation with mouse anti-VSV tag IgG antibody P5D4 (1:10; Boehringer Mannheim, Mannheim, Germany), followed by Alexa 488–conjugated goat anti-mouse IgG (1:200; Molecular Probes, Eugene, OR), both for 45 min. Also, anti-HS antibody JM403 (26) (1:400) was used, which was visualized using Alexa 488–conjugated goat anti-mouse IgM (1:200; Molecular Probes). After each antibody incubation, cryosections were washed three times for 5 min with PBS that contained 0.1% (vol/vol) Tween-20. Finally, cryosections were fixed in 100% (vol/vol) ethanol for approximately 10 s, air-dried, and embedded in 10% (wt/vol) mowiol (Calbiochem). Reactivity of heparinase III was evaluated further by incubation of cryosections with anti-HS stub antibody 3G10 (1:100; Seikagaku, Tokyo, Japan), which was visualized using Alexa 488–conjugated goat anti-mouse IgG.

To study the effect of neuraminidase, we used Sambucus nigra lectin (1:1000; Vector Laboratories, Burlingame, CA), which preferentially binds α2,6-linked neuraminic acid (Neu5Acα2-6Gal), and Peanut agglutinin lectin (1:1000; Vector Laboratories), which binds to an epitope that becomes available after removal of neuraminic acid (Galβ1-3GalNAc). Cryosections first were blocked with streptavidin (1:1000; Sigma), followed by biotin–avidin (1:1000; Sigma), both for 15 min. This was followed by incubation with biotin–labeled lectin for 1 h and Alexa 488–conjugated streptavidin (1:200; Molecular Probes) for 45 min.

Figure 1. Evaluation of the activity of heparinase III and neuraminidase in situ. Renal heparan sulfate (HS) and neuraminic acid expression were demonstrated by immunofluorescence staining of rat cryosections before and after enzyme treatment. Staining for HS (antibody HS4C3) was completely abolished by pretreatment of kidney sections with 0.02 IU heparinase III/ml (A). Neuraminic acid staining with Sambucus nigra (SNA) lectin was abolished after preincubation with 0.04 IU neuraminidase/ml (B). Bar = 50 μm; magnification is identical for each photograph.
Staining for the HSPG core protein agrin was performed using antibody M91 (1:800), which was visualized using CY3-conjugated goat anti-hamster IgG (1:800; Jackson ImmunoResearch, Suffolk, UK). Expression of the podocyte-associated, neuraminic acid–containing protein podocalyxin was studied using antibody ASD-86 (culture supernatant 1:20; provided by the Department of Pathology, Radboud University Medical Centre Nijmegen, Nijmegen, The Netherlands), which was visualized using Alexa 488–conjugated goat anti-mouse IgG. Staining of the sections was examined using a Leica CTR6000 microscope (Leica Microsystems GmbH, Wetzlar, Germany).

**General Histology**
Paraffin-embedded kidneys of the injected rats were sliced into 5-μm sections. After deparaffinization, sections were incubated with DelafIELD’s hematoxylin (Merck, Darmstadt, Germany) for 20 min, followed by incubation with 0.3% (wt/vol) eosin (Merck) in 96% ethanol for 30 s. After dehydration in 100% (vol/vol) ethanol and xylene, sections were incubated in entallan (Merck). Staining of the sections was examined using a Leica CTR6000 microscope.

**Electron Microscopy**
To visualize GAG, we applied the critical electrolyte concentration method as described previously (27). Kidney tissue was fixed overnight in 25 mM sodium acetate (pH 5.6) that contained 2.5% (vol/vol) glutaraldehyde, 0.2 M MgCl₂ and 0.2% (wt/vol) cupromeric blue (Seikagaku, Tokyo, Japan). Tissue was washed three times for 10 min with the same solution without cupromeric blue and three times for 10 min with distilled water that contained 1% (wt/vol) Na-tungstate. Tissue was dehydrated in ascending concentrations of ethanol, the 30 and 50% concentrations containing 1% Na-tungstate, followed by two incubations with 100% (vol/vol) ethanol and xylene, sections were embedded in entallan (Merck). Staining of the sections was examined using a Leica CTR6000 microscope.

**Urine and Serum Analyses**
In all animals, urinary creatinine, GAG, neuraminic acid, protein, and N-acetyl-β-D-glucosaminidase activity were determined in timed urine collections. Serum creatinine was determined in blood that was obtained at the time of killing.

**Creatinine.** Urinary and serum creatinine were measured enzymatically as described by Suzuki and Yoshida (28), using the Aeroset apparatus from Abbott (Hoofddorp, The Netherlands). All reagents were obtained from Roche Diagnostics (Almere, The Netherlands). Creatinine clearance (ml/min) was calculated by the following formula: \([\text{urinary creatinine (μmol/ml)}/\text{serum creatinine (μmol/ml)}] \times [\text{urinary volume (ml)/time (min)}]\).

**Glycosaminoglycans.** Urinary GAG were determined by agarose gel electrophoresis, which separates GAG on basis of backbone structure. Staining was by combined azure A–silver treatment as described by van de Lest et al. (29).

**Neuraminic Acid.** Urinary neuraminic acid was measured enzymatically using a colorimetric assay (Roche, Almere, The Netherlands) according to the manufacturer’s protocol and was expressed as the ratio between urinary neuraminic acid and urinary creatinine (μmol neuraminic acid/mmol creatinine).

**Proteins.** A qualitative examination of proteins in the urine of the injected rats was carried out by SDS-PAGE followed by Coomassie brilliant blue staining as described by Laemmli et al. (30). As a control, a urine sample of a rat with tubular dysfunction (provided by Suzanne Heemskerk, Department of Pharmacology and Toxicology, Nijmegen Centre for Molecular Life Sciences, Nijmegen, The Netherlands) was included. This rat was administered an intraperitoneal injection of 5 mg/kg body wt of the endotoxin LPS and killed after 48 h.

Urinary albumin was analyzed quantitatively using an ELISA kit (Bethyl Laboratories, Montgomery, TX) according to the manufacturer’s protocol. Urinary albumin was expressed as the ratio between urinary albumin and urinary creatinine (mg albumin/mg creatinine).

**N-acetyl-β-D-Glucosaminidase Activity.** Urinary activity of N-acetyl-β-D-glucosaminidase, which is a marker for proximal tubular dysfunction, was determined using the MultiPROBE II apparatus. Urine was incubated with 12.5 mM 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (Sigma) in 0.1 M citric acid (pH 4.4) for 10 min at 37°C. The enzymatic reaction was stopped by the addition of 0.5 M glycine buffer (pH 10.6) that contained 0.025% (vol/vol) Triton, and fluorescence was measured. Urinary N-acetyl-β-D-glucosaminidase activity was expressed as the ratio between urinary N-acetyl-β-D-glucosaminide and urinary creatinine (nmol N-acetyl-β-D-glucosaminide/h per μmol creatinine).

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**Table 1. Experimental setup**

<table>
<thead>
<tr>
<th>Enzyme Solution</th>
<th>Time of Injection</th>
<th>Time of Killing</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 IU heparinase III in PBS</td>
<td>(t = 0\ h) and (t = 24\ h)</td>
<td>(t = 48\ h)</td>
</tr>
<tr>
<td>PBS</td>
<td>(t = 0\ h) and (t = 24\ h)</td>
<td>(t = 48\ h)</td>
</tr>
<tr>
<td>0.4 IU heparinase III in PBS</td>
<td>(t = 0\ h) and (t = 8\ h)</td>
<td>(t = 24\ h)</td>
</tr>
<tr>
<td>0.4 IU neuraminidase in PBS</td>
<td>(t = 0\ h) and (t = 8\ h)</td>
<td>(t = 24\ h)</td>
</tr>
</tbody>
</table>

IU definition: Amount of enzyme that will produce 1 μmol of product per minute under specified conditions of buffer, pH, and temperature.
Results
Glomerular HS and Neuraminic Acid Are Removed after Heparinase III and Neuraminidase Injection, Respectively

Immunofluorescence Studies. To evaluate whether heparinase III had removed HS in vivo, we investigated the presence of HS in the kidney by immunofluorescence staining. Injection of heparinase III into rats resulted in disappearance of staining of renal tissue as demonstrated with the phage display–derived anti-HS antibodies HS4C3 and EW3D10 (Figure 2, A and B), which both recognize sulfated HS domains. In addition, absence of staining was observed with anti-HS antibody JM403 (Figure 2C), which defines a poorly/nonsulfated HS epitope that contains N-unsubstituted glucosamine and d-glucuronic acid residues (26). With anti-HS stub antibody 3G10, a strong staining was found, further indicating that heparinase III had cleaved HS (Figure 2D). Taken together, these data strongly indicate that regeneration is not taking place within the time frame of the experiment. Neuraminidase had no influence on the staining profile. Expression of the core protein agrin, the major HSPG in the GBM, was unaltered (Figure 2E). These data indicate that GBM HS but not the core protein of HSPG was removed by heparinase III treatment.

With Peanut agglutinin lectin, which binds to the galactose-N-acetylgalactosamine (Galβ1–3GalNAc) sequence that is un-

Figure 2. Renal HS expression demonstrated by immunofluorescence staining in rats that were administered injections of PBS, heparinase III, or neuraminidase. Glomerular staining with the anti-HS antibodies HS4C3 (A), EW3D10 (B), and JM403 (C) was abolished after heparinase III injection (A’ through C’). Anti-HS stub antibody 3G10 (D), reflecting heparinase III reactivity, stained only heparinase III–treated kidneys (D’). Heparinase III has thus removed renal HS. Expression of the HS proteoglycan (HSPG) core protein agrin, visualized using antibody MI91 (E), was not affected by either heparinase III (E’) or neuraminidase (E”) treatment. Bar = 50 μm; magnification is identical for each photograph.
masked after removal of neuraminic acid, a glomerular staining was revealed in neuraminidase-treated rats, in contrast to PBS-and heparinase III–treated rats (Figure 3A). These results indicate that neuraminidase had cleaved glomerular neuraminic acid. However, with *Sambucus nigra* lectin, which stains predominantly 2,6-linked neuraminic acid, no aberrant staining could be detected (data not shown). Expression of podocalyxin (a podocyte-associated, neuraminic acid–containing protein) was unaltered after neuraminidase as well as heparinase III injection (Figure 3B).

**Electron Microscopic Studies.** Presence of GAG in the glomerulus was investigated further by cupromeronic blue staining, which revealed a dense network of fine filaments in the GBM. Each filament represents a PG with the GAG chains collapsed onto the core protein (27). The PG filaments that are associated with the podocytic site of the GBM appeared larger and more electron dense in comparison with the PG filaments at the endothelial site. After heparinase III injection, GAG staining of the GBM was almost completely negative, both at the podocytic and endothelial sites (Figure 4). A few short filaments were left, which may be due to the presence of some other GAG (*e.g.*, chondroitin sulfate). As expected, neuraminic acid cleavage by neuraminidase did not result in an altered cupromeronic blue staining of the glomerular capillary wall, because only GAG are visualized by the described method.

By light microscopy, no alterations in renal morphology of the rats that were administered injections of heparinase III or neuraminidase were observed (Figure 5). In addition, electron microscopy revealed that heparinase III injection did not result in an aberrant glomerular ultrastructure. A normal capillary wall was observed, *viz.* a GBM with the fenestrated endothelial cells at the inner side and the podocyte foot processes, interconnected by slit diaphragms, at the outer side. Notably, there was no effacement of podocyte foot processes (Figure 6).

**HS and Neuraminic Acid Are Excreted in the Urine after Heparinase III and Neuraminidase Injection, Respectively**

With the use of agarose gel electrophoresis, HS was found in the urine directly after the first injection of heparinase III, indicating that the enzyme was active and had liberated HS (Figure 7A). Some additional HS was secreted in the urine after the second injection (*t* = 8 to 12 h). No GAG were found in the urine of the rats that were administered PBS and at any time point. In the rats that were administered neuraminidase, levels of free neuraminic acid detected in the urine clearly were increased at all time points tested compared with the rats that were administered PBS and heparinase III (Figure 7B). From these data, it was concluded that HS and neuraminic acid, released by heparinase III and neuraminidase, respectively, were excreted in the urine. No major differences in urinary volume (PBS 10.7 ± 0.9 ml/24 h [*n* = 4], heparinase III 10.3 ± 1.1 ml/24 h [*n* = 4], neuraminidase 8.2 ± 1.1 ml/24 h [*n* = 2]) or creatinine clearance (PBS 2.3 ± 0.3 ml/min [*n* = 4], heparinase III 2.4 ± 0.4 ml/min [*n* = 4], neuraminidase 2.8 ± 0.3 ml/min [*n* = 2]) were found.

**Cleavage of HS Does Not Result in Proteinuria, whereas Cleavage of Neuraminic Acid Does**

SDS-PAGE of urine samples followed by protein staining with Coomassie brilliant blue revealed a substantial increase in urinary albumin in the rats that were administered neuraminidase but not in the rats that were administered heparinase III. The proteinuria likely was of glomerular origin because no low molecular weight proteins, typical for urine of rats with tubular dysfunction, were detected in the urine of the rats that were administered neuraminidase (Figure 8A). Furthermore, we did not detect elevated values of urinary N-acetyl-β-d-glucosaminidase activity, which are increased in case of tubular dysfunction (data not shown). With the use of electron microscopy, no aberrant tubular morphology was observed (Figure 9A). The...
tubular cells, basement membranes, and labyrinth were not affected. Also, a normal proximal tubular brush border was observed (Figure 9B).

To quantify the amount of albumin secreted, we used an ELISA. No increase in urinary albumin could be detected in the rats that were administered heparinase III (e.g., 12 to 24 h 1.1 ± 0.2 versus 1.4 ± 1.1 mg albumin/mg creatinine for rats that were administered heparinase III and PBS, respectively; Figure 8B). On the contrary, injection of neuraminidase into rats was found to cause significant albuminuria (e.g., 12 to 24 h 13.6 ± 0.3 mg albumin/mg creatinine).

Discussion
This study evaluates the role of HS in glomerular permeability using a highly controlled in vivo approach. Removal of GBM HS after cleavage by heparinase III was checked at three levels: By immunofluorescence using antibodies to various (sulfated) HS domains, by electron microscopy using cupromeronic blue staining according to the critical electrolyte concentration method, and by analysis of HS in the urine. A positive control for proteinuria was included, viz. treatment with neuraminidase, which has been reported to induce proteinuria in mice by removal of neuraminic acid from the glomerular filtration barrier (22). Using the in vivo approach, we indeed found that removal of glomerular neuraminic acid results in acute albuminuria. Removal of HS from the GBM did not result in acute proteinuria. These results therefore indicate that HS in the GBM does not play a major, direct role in the glomerular filtration of proteins.

Various studies suggest a crucial role for HS in glomerular permeability. Intravenous injection of rats with the monoclonal anti-HS antibody JM403 resulted in a dosage-dependent increase in albuminuria, which was maximal within 2 h after injection and decreased thereafter (17). This antibody defines an HS epitope that contains one or more N-unsubstituted glu-
cosamine and d-glucaric acid residues. l-Iduronic acid and O-sulfate residues seem to inhibit JM403 binding to HS (26). Therefore, the albuminuria observed may not be due to blockade of the negatively charged sulfate groups of HS, which have been reported to be responsible for glomerular permselectivity.

Other studies have shown that kidney perfusion with heparinase III resulted in proteinuria. In their seminal studies, Kanwar et al. (18,19) reported that treatment of isolated rat kidneys with heparinase III resulted in an increase of native ferritin (440 kD) in all layers of the GBM and even in the urinary space. Treatment with heparinase III also resulted in an increased permeability of the GBM to BSA (68 kD) (19,20). However, from the study of Jeansson et al. (21), it is unclear whether HS plays a role in glomerular filtration. In the studies mentioned, there was no thorough check for whether HS was removed completely after heparinase III treatment, and (generally) isolated kidneys were used. In our study, we used intravenous injection of heparinase III, which may represent a more physiologic approach to study the role of HS in charge-selective permeability.

It still is not clear whether a decrease in GBM HS that is seen in patients with renal disease is a cause of or a result from proteinuria. Our study indicates that removal of HS from the GBM does not result in albuminuria. A number of proteinuric renal diseases have been reported without aberrant GBM HS expression, including IgA nephropathy (14), congenital nephrotic syndrome of the Finnish type (31,32), and puromycin aminonucleoside nephrosis (19).

There already are some additional indications that HS does not play a major role in the charge-selective barrier properties of the GBM. In podocyte-specific agrin knockout mice, no proteinuria was observed (33), which also was the case for mice...
that lack exon 3 of perlecan (which codes for the HS-bearing domain), although this HSPG is not the major GBM HSPG (34). However, Morita et al. (35) reported that Hspg2Δ3/Δ3 mice show higher levels of proteinuria than do wild-type mice after intraperitoneal injection of BSA. GBM HS, however, did not seem to be affected, and the degenerative changes that were observed in podocytes (e.g., fusion of foot processes) and endothelial cells may have contributed to the proteinuria. Note that in our study we did not challenge rats with an overload of protein.

Transgenic mice that overexpress human heparanase (endo-β-d-glucuronidase that catalyzes the hydrolytic cleavage of HS) were reported to have only a slightly elevated level of protein in the urine (36). Nevertheless, Levidiotis et al. (37) found that heparanase contributes to the pathogenesis of proteinuria in passive Heymann nephritis. The effect on HS in the GBM, however, was not analyzed, and the mechanism through which heparanase contributed to proteinuria remains to be established.

**Conclusion**

Removal of HS from the GBM using a highly controlled in vivo rat model does not result in acute proteinuria, indicating that HS is not a major determinant for the charge-selective characteristics of the glomerular capillary wall.

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Figure 9. Electron micrographs of proximal tubules from rats that were administered injections of PBS, heparinase III, or neuraminidase. No abnormalities were observed in the rats that were administered injections of heparinase III and neuraminidase (A). The brush border also appeared normal (B). Bar = 500 nm; magnification is identical for each photograph.

Disclosures

None.

References

18. Kanwar YS, Linker A, Farquhar MG: Increased permeability of the glomerular basement membrane to ferritin after


See the related editorial, “Breaking Down the Barrier: Evidence against a Role for Heparan Sulfate in Glomerular Permselectivity,” on page 672–674.